

**STRATEGIES TO ENHANCE THE SAFETY OF FRESH
PRODUCE AND IMPROVE THE MICROBIAL QUALITY OF
FRUIT JUICE**

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STRATEGIES TO ENHANCE THE SAFETY OF FRESH PRODUCE AND IMPROVE THE MICROBIAL QUALITY OF FRUIT JUICE

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Recent outbreaks of produce-related foodborne illness have increased consumer awareness of the potential health risks associated with the consumption of fresh leafy greens. Additionally, microbial spoilage of fruit juice and juice beverages through contamination with *Alicyclobacillus* spp. has caused significant economic losses worldwide.

The first part of the study focused on methods to enhance the safety of minimally processed green onions and baby spinach. The individual and combined inactivation efficacies of ultraviolet light (254 nm), acidified sodium chlorite (ASC), and mild heat treatments were evaluated and optimized for surface and infiltrated *E. coli* O157:H7 contamination on green onions and baby spinach. Due to the limited efficacies of these methods for infiltrated pathogens, a novel method utilizing gaseous antimicrobial ethyl pyruvate (EP) was proposed.

In the second part of the study, the distribution of *Alicyclobacillus* spp. allelic types (ATs) collected from fruit juices, beverages, ingredients, and environmental samples over a period of ten years was analyzed. Our results suggest a predisposition for certain ATs of *Alicyclobacillus* spp. depending on the juice or ingredient isolation source. A further characterization of the thermal resistance, guaiacol production, and genetic properties of *Alicyclobacillus acidoterrestris*, the most common *Alicyclobacillus* species implicated in juice spoilage, was investigated. This

information will assist in controlling *Alicyclobacillus* spp. contamination through the development of thermal processing regimes tailored to the relevant *Alicyclobacillus* spoilage organisms in different juice and beverage products, while avoiding excessive processing that would lead to diminished quality in the final product.

BIOGRAPHICAL SKETCH

M. Zeki Durak was born and raised in Eskisehir, Turkey. He graduated from the Department of Food Engineering at Akdeniz (Mediterranean) University in 2003 with an honors degree. During his undergraduate studies, he received a research fellowship from The Scientific and Technological Research Council of Turkey (TUBITAK) and worked on developing sodium alginate and sodium caseinate-based edible films for extending the shelf life of mushrooms. Upon graduation from Akdeniz University, he joined the Cornell University Department of Food Science as a visiting fellow, where he worked on projects related to the shelf-life extension of pasteurized milk under the supervision of Dr. Kathryn J. Boor. In 2004, he started his M.S. studies at Cornell University and conducted research on developing biosensors utilizing photographic silver halide amplification and liposomal delivery with Dr. Joseph H. Hotchkiss. After completing his M.S., he continued with his Ph.D. studies with Dr. Randy W. Worobo also at Cornell, with a research focus on enhancement of fresh produce safety and improvement of microbial fruit juice quality.

To my beloved family and wife

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CHAPTER 1

INTRODUCTION

FOODBORNE ILLNESSES

Foodborne diseases continue to be a significant public health problem worldwide in the 21st century, as well as a major cause of illnesses and deaths in the United States. In a recent study, Scallan *et al.* (2011) estimated that 31 foodborne pathogens acquired in the United States caused an estimated 9.5 million episodes of foodborne illnesses, resulting in 55,961 hospitalizations and 1,351 deaths annually (42). The pathogens responsible for most of the illnesses included norovirus (5.5 million, 58% of total illnesses), non-typhoidal *Salmonella* spp. (1.0 million, 11% of total illnesses), *C. perfringens* (1.0 million, 10% of total illnesses), and *Campylobacter* spp. (0.8 million, 9% of total illnesses) (Table 1.1). The leading causes of hospitalizations were non-typhoidal *Salmonella* spp. (34.6%), norovirus (26%), *Campylobacter* spp. (15.1%) while the leading causes of death as non-typhoidal *Salmonella* (28%), *L. monocytogenes* (19%), and norovirus (11%) (Table 1.2). The World Health Organization (WHO, 1997) estimated that diseases caused by the major foodborne pathogens cost up to 35 billion dollars annually in medical costs and lost productivity in the United States. In a recent study, Roberts (2007) estimated an annual cost of all foodborne disease in the United States to be \$1.4 trillion based on the willingness-to-pay calculation (WTP) which also includes pain and suffering, lost leisure time, and disruption of daily activities (31, 41).

Due to the increased worldwide population, there is a higher demand for more food, but the food must be affordable for all consumer income levels. Centralized, industrial-scale systems that include large scale agricultural farming and animal

production, increases food production and control prices. Nearly all food consumed in developed countries today is produced using modern intensive farming practices (45). Additionally, globalized trade and distribution allows food to travel around the world. This new way of accessing the food has brought many benefits to consumers such as year-round availability, competitive prices, and consistent quality. However, the large scale centralized production and global trade has brought some disadvantages. Foodborne illness outbreaks spread through large geographic areas very quickly and affect the health of people in numerous countries at the same time. These types of outbreaks often result in a high number of cases and increase the chances that an outbreak will be detected (46). Globalization is not the only reason foodborne illnesses occur. Nyachuba (2010) reported five trends affecting the occurrence of foodborne illnesses: 1) large-scale production and wide distribution of food; 2) globalization of food supply; 3) eating outside of the home, 4) microbial genomic diversification/emergence of new pathogens; and 5) growing population of at risk consumers (38).

According to a comprehensive literature survey of over 1,400 different species of human foodborne pathogens, 58% were found to be zoonotic (48). Another survey reported 60.3% of Emerging Infectious Diseases (EIDs) events were caused by zoonotic pathogens (32). Woolhouse and Gowtage-Sequeria (2005) identified 10 main factors that affect emerging, reemerging pathogens, and “changes in land use or agricultural practices” was ranked #1 as the most significant factor (48). The emergence of drug-resistant *Salmonella* serotypes over the past decades is considered to be a response to intensive farming and antibiotic use in farm animals (47). A recent genomic analysis of the *E. coli* O157:H7 strain (TW14359) associated with the 2006 spinach outbreak has revealed a unique 70 kb DNA fragment that contains genes

which may have enhanced the virulence of the pathogen, including its adaptation to attach and survive on plants (34). Another major produce related outbreak in Germany and France was caused by a rare *E. coli* serotype O104:H4. This newly emerged pathogen had an unusual combination of pathogenic factors coming from entero-aggregative *E. coli* in addition to producing Shiga toxin (Stx) (44). This Stx-producing serotype O104:H4 has not been detected in animals and has rarely been associated with causing HUS. This serotype lacked intimin (*eae*), which is responsible for adhesion to epithelial cells in EHEC (36). Because of its hybrid pathogenicity characteristics from pathotypes EAEC and EHEC, Brzuszkiewicz *et al.* (2011) assigned a new pathotype of Entero-Aggregative-Haemorrhagic *Escherichia coli* (EAHEC) (2).

Due to frequent occurrence of worldwide foodborne illness (FBI) outbreaks, many countries have food safety programs and organizations in place. In the United States, many agencies are involved in regulating, enforcing and supporting the food safety system. Some of these groups include the United States Department of Agriculture-Food Safety and Inspection Service (USDA-FSIS), the US Food and Drug Administration-Center for Food Safety and Applied Nutrition (FDA-CFSAN), Centers for Disease Control and Prevention (CDC), Environmental Protection Agency (EPA) and many other state/city/county departments of health (38). Beyond the US, the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) in the EU and as well as World Health Organization (WHO) have focused the efforts of keeping the World's food safe. Despite all the global efforts to prevent foodborne illnesses, foodborne outbreaks continue to occur (Table 1.3).

Table 1.1. Estimated annual number of episodes of domestically acquired foodborne illnesses caused by 15 selected bacterial and viral pathogens in the US, The data was adopted from Scallan *et al.* (42).

Pathogen	Laboratory confirmed	Multipliers			Travel Related, %	Foodborne, %	Mean illnesses, (% Total illnesses)
		Under-reporting	Under-diagnosis				
<i>Bacillus cereus</i>	85	25.5	29.3	<1	100	63,400 (0.7%)	
<i>Brucella</i> spp.	120	1.1	15.2	16	50	839 (0.01%)	
<i>Campylobacter</i> spp.	43,696	1	30.3	20	80	845,024 (9%)	
<i>Clostridium perfringens</i>	1,295	25.5	29.3	<1	100	965,958 (10%)	
STEC O157	3,704	1	26.1	4	68	63,153 (0.7%)	
STEC non-O157	1,579	1	106.8	18	82	112,752 (1.2%)	
ETEC, foodborne	53	25.5	29.3	55	100	17,894 (0.2%)	
Diarrheagenic <i>E. coli</i> other than STEC and ETEC	53	25.5	29.3	<1	30	11,982 (0.1%)	
<i>Listeria monocytogenes</i>	808	1	2.1	3	99	1,591 (0.02%)	
<i>Salmonella</i> spp., nontyphoidal	41,930	1	29.3	11	94	1,027,561 (11%)	
<i>S. enterica</i> serotype Typhi	433	1	13.3	67	96	1,821 (0.02%)	
<i>Shigella</i> spp.	14,864	1	33.3	15	31	131,254 (1.4%)	
<i>Yersinia enterocolitica</i>	950	1	122.8	7	90	97,656 (1%)	
Hepatitis A	3,576	1.1	9.1	41	7	1,566 (0.02%)	
Norovirus	NA	NA	NA	<1	26	5,461,731 (58%)	

Table 1.2. Estimated annual number foodborne hospitalization and deaths caused by 15 selected pathogens, US. The data and table was adopted from Scallan *et al.* (42)

Pathogen	Hospitalization rate, %	Hospitalization, mean (%)	Death rate, %	Death, mean (%)
<i>Bacillus cereus</i>	0.4	20 (0.03%)	0	0 (0%)
<i>Brucella</i> spp.	55	55 (0.1%)	0.9	1 (0.07%)
<i>Campylobacter</i> spp.	17.1	8,463 (15.1%)	0.1	76 (5.6%)
<i>Clostridium perfringens</i>	0.6	438 (0.8%)	<0.1	26 (1.9%)
STEC O157	46.2	2,138 (3.8%)	0.5	20 (1.5%)
STEC non-O157	12.8	271 (0.5%)	0.3	0 (0%)
ETEC, foodborne	0.8	12 (0.02%)	0	0 (0%)
Diarrheagenic <i>E. coli</i> other than STEC and ETEC	0.8	8 (0.01%)	0	0 (0%)
<i>Listeria monocytogenes</i>	94	1,455 (2.6%)	15.9	255 (18.9%)
<i>Salmonella</i> spp., nontyphoidal	27.2	19,336 (34.6%)	0.5	378 (28%)
<i>S. enterica</i> serotype Typhi	75.7	197 (0.35%)	0	0 (0%)
<i>Shigella</i> spp.	20.2	1,456 (2.6%)	0.1	10 (0.74%)
<i>Yersinia enterocolitica</i>	34.4	533 (1%)	2	29 (2.1%)
Hepatitis A	31.5	99 (0.18%)	2.4	7 (0.5%)
Norovirus	0.03	14,663 (26.2%)	<0.1	149 (11)

In order to develop effective FBI outbreak control strategies for emerging, reemerging and reoccurring pathogens, it is imperative to understand pathogen transmission sources as well as identification and association of both the contaminated food items and the responsible pathogens. This critical information would help food inspectors to traceback to the origin of contamination and develop better trainings and intervention technologies. The CSPI has compiled FBI outbreak data from 1999 to 2008 which identifies the major bacterial and viral etiologies associated with the same food category more than 5 outbreaks (Table 1.4). According to CSPI's data, *Campylobacter* spp., *Salmonella* spp., and *Escherichia coli* O157:H7, and norovirus have been identified as frequent pathogens resulting in FBI outbreaks (Table 1.4). Norovirus and *Salmonella* spp. were found to be the most prevalent etiologies of produce and multi-ingredient food related outbreaks, while *E. coli* O157:H7 remained as a second most important etiological bacterial foodborne pathogen associated with produce/leafy greens related FBI between 1999-2008 (21). However, *Salmonella* spp. was the most common pathogen associated with produce outbreaks, followed by *E. coli* O157:H7, *Shigella*, and *Campylobacter*, while the most common virus was hepatitis A followed

by norovirus between 1973-1997 (43). On the other hand, *E. coli* O157:H7 was and has been identified as the number one etiological agent of FBI outbreaks associated with ground beef (21, 23) (Table 1.4). Here, we will review several frequent FBI associated microorganisms.

Salmonella spp. are an important cause of gastrointestinal illness, salmonellosis, in humans. The genus *Salmonella* contains more than 2300 serotypes, all of which are considered to be human pathogens. Salmonellosis is characterized by developing symptoms of diarrhea, fever, and abdominal cramps 12 to 72 hours after infection and usually lasts for 4-7 days. *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most frequently reported non-typhoidal serotypes associated with outbreaks in many countries. Animals serve as the primary reservoir for nontyphoid *Salmonella* serotypes and antibiotic use in feed animals may have resulted in the emergence of antibacterial resistant serotypes (25). Norovirus is usually transmitted from human to human. However, some norovirus strains may infect humans, pigs, cattle, and mice. Thus, the transmission of infection exists from animal to human. The infective dose of the virus is considered to be very low (10-100 virus particles) and can be spread via aerosols. Foodborne outbreaks usually have been associated with infected food handlers or food contaminated by human feces. While norovirus is the microorganism most often associated with foodborne illnesses, it is still considered to be under reported (Table 1.1). The incidence of illnesses is highest in children, but the illness also occurs in adults. Norovirus infection is usually associated with nausea, vomiting, and diarrhea lasting 20-40 hours.

Table 1.3. Selected recent bacterial foodborne outbreaks, 2006-2011. The outbreaks information was collected from CDC, FDA, EFSA, selected research papers, other governmental or institutional sources (4-8, 10-18, 27-29, 39).

Year	Pathogen	Number of cases	Number of deaths	Affected region	Source
2011	<i>E. coli</i> O104:H4	3929	47	Germany and France (EU)	Unknown
2011	<i>Listeria monocytogenes</i>	72	13	Multistate (US)	Rocky Ford-brand cantaloupes
2011	<i>Salmonella</i> Agona	106	0	Multistate (US)	Papayas imported from Mexico
2010	<i>Salmonella</i> serotype 4, 5, 12:i:-	140	0	Multistate (US)	Alfalfa sprouts
2010	<i>Salmonella</i> Enteritidis	1939	0	Multistate (US)	Shell eggs
2009	<i>Salmonella</i> Typhimurium	714	9	Multistate (US)	Peanut butter
2009	<i>Salmonella</i> Saintpaul	235	0	Multistate (US)	Raw alfalfa sprouts
2009	<i>E. coli</i> O157:H7	72	0	Multistate (US)	Prepackaged cookie dough
2008	<i>Salmonella</i> Saintpaul	1442	2	Multistate (US)	Jalapeno and serrano peppers from Mexico
2008	<i>Salmonella</i> Litchfield	51	0	Multistate (US)	Cantaloupes from Honduras
2007	<i>Shigella sonnei</i>	227	0	Denmark, Australia (EU)	Baby corn from Thailand
2007	<i>Salmonella</i> Senftenberg	55	0	England, Scotland, Denmark, the Netherlands (EU), and US	Fresh Basil from Israel
2007	<i>Salmonella</i> serotype 4, 5, 12:i:-	401	0	Multistate (US)	ConAgra frozen pot pies
2007	<i>Listeria monocytogenes</i>	5	3	Massachusetts (US)	Pasteurized local farm milk
2007	<i>E. coli</i> O157:H7	40	0	Multistate (US)	Topp's brand ground beef patties
2006	<i>E. coli</i> O157:H7	199	3	Multistate (US)	Dole baby spinach
2006	<i>Salmonella</i> Typhimurium	183	0	Multistate (US)	Tomatoes
2006	<i>E. coli</i> O157:H7	81	0	Multistate (US)	Lettuce in Taco John's restaurants
2006	<i>E. coli</i> O157:H7	71	0	Multistate (US)	Lettuce in Taco Bell restaurants

Hepatitis A virus (HAV) is an obligate human pathogen and is not found in animal reservoirs. The transmission of HAV is by the fecal-oral route (40). Similar to norovirus, most reported HAV outbreaks have been linked to infected handlers at or close to point of service, but other modes of transmission can occur. Symptoms of HAV infection include diarrhea, jaundice, fever, abdominal pain, loss of appetite, and dark urine (40). HAV is a self-limiting disease but the duration of the illness is variable from 2 weeks to 3 months. The most common food vehicles associated with norovirus and HAV outbreaks are lettuce, green onions, strawberries, berries, salads, green bell pepper, and sandwiches (19, 22, 35).

Escherichia coli is a member of the intestinal microbiota of humans and other mammals. However, there are highly adapted *E. coli* strains that have acquired virulence determinants that enable them to cause a broad range of diseases (33). The *E. coli* which cause enteric diseases have been divided into six main categories (pathotypes); enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC). EHEC is one of the most frequent foodborne outbreak associated pathotypes, particularly the O157:H7 serotype, and causes diarrhea-associated hemolytic uremic syndrome (HUS). Although only a small fraction of all *E. coli* O157:H7 disease cases develop HUS, HUS is very severe and is a life threatening disease characterized by red blood cell destruction. The Shiga toxin *E. coli* (STEC) of EHEC refers to those strains of *E. coli* that produce at least 1 member of a class of potent cytotoxins called Shiga toxin (Stx) (30). Another important frequent foodborne associated pathotype is ETEC, which causes watery diarrhea which can range from a mild, self-limiting disease to a severe purging disease (Table 1.2) (33).

Table 1.4. CSPI Foodborne illness outbreak data 1999-2008 more than five outbreaks (FDA and USDA, 1999-2008). The data was taken from CSPI Foodborne Illness Outbreak Data 1999-2008 (21).

Foods	Outbreaks	Cases	Etiology #2 / #3 (outbreaks)
Beverages	N=21	N=1,257	
Juice and cider	21	1257	Other Chemical/Toxin (5) / Norovirus (4); <i>E. coli</i> (4)
Dairy			
Unpasteurized milk & cheese	76	1517	<i>E. coli</i> O157:H7 (11) / <i>Salmonella</i> (6)
Cheese	43	916	<i>Salmonella</i> (10)
Ice cream	42	717	Norovirus (10)
Other dairy	30	1156	
Eggs	N=110	N=3,084	
Eggs and egg dishes	110	3084	<i>Staphylococcus</i> (5) / Norovirus (4)
Produce	N=538	N=20,901	
Greens-based salad	288	8385	<i>E. coli</i> (15); <i>Salmonella</i> (14)
Tomatoes	35	3081	Norovirus (8)
Lettuce	61	1900	<i>E. coli</i> O157:H7 (14) / <i>Salmonella</i> (4)
Melon	36	1855	Norovirus (13)
Chill peppers	11	1715	<i>Salmonella</i> (2)
Fruit salad and mixed fruits	43	1595	<i>Salmonella</i> (5)
Sprouts	25	762	<i>E. coli</i> (5)
Salsa	39	1608	<i>Salmonella</i> (11)
Multi-ingredient Foods	N=188	N=4,500	
Potato salad	43	1772	<i>Salmonella</i> (5); <i>Staphylococcus</i> (5)
Beans, legumes	67	1372	<i>Bacillus</i> (12) / <i>Salmonella</i> (9)
Pasta salad	20	412	<i>Salmonella</i> (2) / <i>Staphylococcus</i> (1); <i>Clostridium</i> (1)
Coleslaw	23	579	<i>Staphylococcus</i> (3) / <i>Bacillus</i> (2); <i>Salmonella</i> (2)
Fried rice	35	365	
Beef	N=435	N=9,986	
Other beef and beef dishes	258	7245	Norovirus (34); <i>Salmonella</i> (32)
Ground beef	177	2741	Norovirus (22) / <i>Clostridium</i> (20)
Poultry	N=514	N=12,980	
Chicken and chicken dishes	380	7567	Norovirus (73) / <i>Clostridium</i> (68)
Turkey	134	5413	<i>Salmonella</i> (40) / Norovirus (31)

FRESH PRODUCE RELATED FOODBORNE ILLNESSES

The consumption of fruits and vegetables is increasing and becoming an important component of a healthy diet as consumers' awareness increases with the potential health benefits of fruits and vegetables (3). The fresh cut and minimally processed prepackaged fruits and vegetables are a growing sector because they combine these healthful characteristics with convenience. As a result, a greater variety of prewashed ready-to-eat and minimally processed fruits and vegetables have been introduced into the retail market over the past decade in order to meet consumer demands for healthy and convenient products. Bagged salad is one of the fastest growing food retail product lines in the US and expected to reach to 8.8 billion dollars in market value by

2012. Unfortunately, as sales of fresh cut and minimally processed fresh produce have increased, so have foodborne illnesses associated with these products. The proportion of foodborne outbreaks attributed to produce as a vehicle has increased dramatically from 0.7% of all foodborne outbreaks in the 1970's to 6% in the 1990's and 13% between 1990-2005 (24, 43). The Center for Science in the Public Interest (CSPI) identified a total of 684 produce-related foodborne illness outbreaks resulting in 26,735 illnesses between 1998-2007 (20). However, there is no clear evidence that the incidence of increasing produce related outbreaks is only due to the increase in the fresh produce consumption or associated with any single factor. Investigations of the recent produce-related outbreaks indicate that the contamination routes of pathogens have not been adequately controlled during pre-harvest, harvest, and post-harvest level. While consumers demand healthier and more nutritious food, safety concerns directly impact consumers' behaviors. According to the Mintel's (2008) Bagged Salads and Salad Dressings report, bagged salad sales in the US declined 10.5% in 2006 when compared with 2005 sales, due to the baby spinach associated *E. coli* O157: H7 outbreak in 2006 (37). Tracing the original contamination source in produce related outbreaks is very difficult and often results in delays in the identification of the transmission vehicle and the ultimate cause. This may also result in large number of outbreaks in a relatively short time. The challenges in tracing the contamination source is compounded by the relatively short shelf-life of fresh produce, incomplete or discarded records, limited labeling, mixing harvest from different farms, and increased globalization of produce distribution (1, 46). The low prevalence of pathogens in the food also challenges the likely isolation from the suspected food samples. For example, the *Salmonella* Saintpaul outbreak which resulted in 1442 illnesses and 2 deaths was one of the largest produce related outbreaks. It was initially linked to tomatoes based on the epidemiological data (9). However, the failure of recovering

Salmonella Saintpaul from the sample tomatoes led investigators to reconsider sources. In the end, jalapeño peppers were indicated as the major source of contamination as well as serrano peppers (10). Epidemiological data from the 2010 STEC *E. coli* O104:S4 outbreak in Europe indicated salads and raw vegetables as the source of the contamination. Later, cucumbers were suspected as a vehicle of transmission, but this could not be confirmed microbiologically. Subsequent epidemiological investigation identified other consumed foods and indicated fenugreek seeds imported from Egypt as the leading suspect but additional evaluation eliminated the fenugreek seeds as the source of the outbreak. However, due to increased number of cases in EU Member States, identification of indistinguishable PFGE patterns of numerous *E. coli* O104:H4 outbreak strains, political and coordination challenges in Europe made the tracking of the contamination source and the association between the outbreaks in different countries a very challenging task (26, 27). Given the facts that the produce related outbreaks frequently occurs all over the world and tracing the contamination source is a challenge, it is essential to understand the risk factors in fresh produce environments and apply GAPs at the production level to enhance the safety of produce.

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CHAPTER 2

EFFICACY OF ULTRAVIOLET (UV) LIGHT, ACIDIFIED SODIUM HYPOCHLORITE, AND MILD HEAT FOR DECONTAMINATION OF SURFACE AND INFILTRATED *Escherichia coli* O157:H7 ON GREEN ONIONS AND BABY SPINACH

ABSTRACT

Produce-associated foodborne illnesses outbreaks have highlighted the need for more effective decontamination methods to ensure the safety of fresh produce. The main objective of this study was to evaluate the individual and combined efficacies of germicidal UV light (12.5 – 500 mJ/cm²), acidified sodium chlorite (ASC; 10 – 200 ppm), and mild heat (40°C – 50°C) for decontaminating green onions and baby spinach inoculated with *E. coli* O157:H7. Samples were inoculated using both spot and dip-inoculation methods to mimic surface and infiltrated *E. coli* O157:H7 contamination, respectively. In green onions and baby spinach, the individual efficacies of UV, ASC, and mild heat treatments varied based on the produce type and contamination method. Following analysis the efficacies of the single treatments, a combined treatment with 125 mJ/cm² UV and 200 ppm ASC at 50°C was selected for spot-inoculated green onions, and a combined treatment with 125 mJ/cm² UV and 200 ppm ASC at 20°C was selected for spot and dip-inoculated baby spinach. While >5 log reduction was achieved with the combination treatment for spot-inoculated green onions with an initial contamination level of 7.2 log CFU/spot, the same treatment reduced *E. coli* O157:H7 populations below the detection limit (<1 log) on green onions spot-

inoculated with at a lower contamination level (4.3 log CFU/spot). On spot and dip-inoculated baby spinach, the combined treatment reduced *E. coli* O157:H7 populations by 2.8 log CFU/spot and 2.6 log CFU/g, respectively. The combined treatment of 500 mJ/cm² UV and 200 ppm ASC at 50°C selected for the decontamination of dip-inoculated green onions resulted in a 2.2 log CFU/g reduction. These findings suggest that when foodborne pathogens contaminate produce and subsequently infiltrate, attach or become localized into protected areas, the individual or combined applications of UV, ASC, and mild heat treatments have limited decontamination efficacies on both green onions and baby spinach (< 3 log). However, treatments combining UV, ASC, and mild heat could be a promising application for reducing pathogen populations (> 5 log) on *E. coli* O157:H7 surface-contaminated green onions. This study also highlights the importance of developing and optimizing produce-specific decontamination methods to ensure the safety of fresh produce commodities.

INTRODUCTION

Outbreaks of foodborne illness associated with fresh produce continue to be a major concern as consumer demand for healthier and non-thermally processed food increases. The proportion of foodborne outbreaks attributed to contaminated produce has risen dramatically from 0.7% of all foodborne outbreaks in the 1970s, to 6% in the 1990s, and 13% from 1990 to 2005 (10, 33). The Center for Science in the Public Interest (CSPI) identified a total of 684 produce-related foodborne illness outbreaks resulting in 26,735 illnesses between 1998 and 2007 (8). The inherent difficulty in tracing the source of contamination in produce-related outbreaks can result in a large number of outbreaks in a relatively short time. Challenges in tracing the contamination

source of an outbreak are common (6, 11, 12) and they are compounded by the relatively short shelf-life of fresh produce, low prevalence of pathogens, incomplete or discarded records, limited labeling on produce, mixing of produce from multiple farms, and increasing globalization in produce distribution (1, 39).

Foodborne pathogens can be introduced onto produce at many points in the farm-to-fork production cycle. These contamination sources may include but are not limited to wild and domestic animals, irrigation and wash water, soil and soil amendments, humans, transportation, processing, packaging, marketing, and the cold chain (39). Contaminating pathogens have been shown to survive on the surface of produce for an extended period of time. It has been reported that *E. coli* O157:H7 is capable of persisting more than 20 days on lettuce plants exposed to pathogens through spray or surface irrigation water (35). Islam *et al.* (2004) reported that *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium were detected on irrigation water-contaminated lettuce and parsley plants for up to 60 and 77 days, respectively, and on compost for up to 177 and 231 days, respectively (20, 21). In addition to the surface contamination of produce, the infiltration of foodborne pathogens through the stomata, root system, seed coat, wounds, or bruises on the surface of a leaf or fruit and subsequent migration and localization throughout the edible portion of the plants has been reported in many studies (10, 13, 16, 26, 34-36). Once pathogens are infiltrated, they are inaccessible to commonly used surface decontamination methods.

Leafy vegetables have been recently associated with large *E. coli* O157:H7 and *Salmonella* spp. outbreaks. In the United States, a multi-state outbreak of *E. coli* O157:H7 infections resulted in 183 cases linked to the consumption of baby spinach (5). CSPI has identified 363 separate outbreaks associated with leafy greens, making

them number one in the FDA's top ten riskiest foods (8). Many treatments have been proposed for the decontamination of leafy greens harboring *E. coli* O157:H7. Currently, chemical disinfectants such as chlorinated water at 50 to 200 ppm concentrations are the most commonly used disinfection methods in commercial produce processing plants. However, these surface treatments have been shown to achieve a less than 2 log reduction in *E. coli* O157:H7 populations (3, 29). Thus, the use of chlorine solutions alone is not effective in reducing or completely inactivating *E. coli* O157:H7 populations on fresh produce.

Investigations of recent and frequent produce-related outbreaks indicate that pathogen contamination routes are not always adequately controlled during the entire production cycle. Thus, this information highlights the continued need to eliminate sources of contamination, understand pathogen-produce interactions, and develop necessary produce and pathogen-specific intervention strategies to enhance the safety of fruits and vegetables. The main objective of this study was to evaluate the effectiveness of individual and combined treatments employing UV light, acidified sodium chlorite (ASC), and mild heat in reducing surface and infiltrated *E. coli* O157:H7 populations on green onions and baby spinach.

MATERIALS AND METHODS

Strains of *E. coli* O157:H7. Initial evaluation of the individual efficacies of UV, ASC, and mild heat treatments was performed using *E. coli* O157:H7 ATCC 43895 (raw hamburger meat isolate). After the optimum parameters were identified for the single treatments, the combined treatments were tested on a cocktail of five *E. coli* strains: O157:H7 ATCC 43889 (clinical isolate), ATCC 43894 (clinical isolate),

ATCC 43895 (raw hamburger meat isolate), 933 (raw hamburger meat isolate) and ATCC 35150 (clinical isolate). All strains were maintained at -80°C with 15% glycerol (Fisher Scientific, Pittsburgh, PA). When needed, *E. coli* O157:H7 strains were cultured overnight at 37°C in Tryptic Soy Broth (TSB). Equal volumes of individual cultures were mixed to form a five-strain cocktail ($\sim 10^9$ CFU/ml).

Inoculation of green onions and baby spinach. Fresh green onions and baby spinach were purchased from a local grocery store (Wegmans, Geneva, NY) one day prior to each experimental trial. Each green onion was trimmed to a length of 20 cm. Green onions and baby spinach leaves were washed three times with deionized (DI) water and dried in a biosafety cabinet for 30 minutes at ambient temperature ($\sim 20^{\circ}\text{C}$). Green onions and baby spinach were inoculated using standard dip and spot-inoculation methods (27). The DI-washed green onions were immersed in a 1:100 or 1:200 dilution of the overnight *E. coli* 43895 or the five-strain cocktail culture in 0.1% peptone water for one minute and dried in a biosafety cabinet for two hours at ambient temperature. For spot-inoculation, 50 μl of *E. coli* O157:H7 ($\sim 10^8$ CFU/ml) culture was applied to the surface of green onions and spinach, and samples were dried in a biosafety cabinet for two hours at ambient temperature.

UV treatment. The spot and dip-inoculated green onions (20 g) and baby spinach leaves (10 g) were placed in sterile aluminum sample trays. The UV box unit ($84 \times 24 \times 26$ cm) specifically designed for these trials consisted of four low pressure germicidal UV (254 nm) lamps (Enaqua, Vista, CA) positioned approximately 15 cm above the sample treatment area. The UV lamps were turned on at least 30 minutes before each treatment to allow the bulbs to reach maximum intensity and stabilize the UV exposure dose output. Samples were treated with UV levels ranging from 12.5 to

500 ±10 mJ/cm². While the spot-inoculated green onions and baby spinach were only exposed to UV light on their contaminated surfaces (one side), the dip-inoculated samples were exposed to the same dose of UV treatment on both sides. The UV exposure dose intensity in the UV box unit was measured using a UVX-25 radiometer (UVP, Upland, CA). To limit the temperature increase inside the UV box and maintain a constant level of UV dose exposure, the UV box was operated in a cold room (4°C). This provided consistency in UV exposure treatments and prevented the inactivation of pathogens due to a temperature increase inside the UV box. The desired UV dose was achieved by altering the exposure time at the fixed distance. The temperature inside the UV box was monitored with a Corning digital probe pH/TempMeter 4 (Glendale, AZ).

Acidified sodium chlorite (ASC) and mild heat treatments. Both spot and dip-inoculated green onions and baby spinach leaves were placed in sterile plastic pouches. Sodium hypochlorite solutions were prepared fresh before each trial and acidified to pH 6 using 10% or 1% lactic acid solutions. The pH of the ASC solutions was measured using a Thermo Scientific Orion 2-Star pH meter (Waltham, MA, USA). The free chlorine concentration in the solutions was measured and monitored using Merckoquant Chloride test strips (Merck, Darmstadt, Germany). An ASC solution (100 ml of water containing 0, 10, 50, 100, or 200 ppm free chlorine at pH 6) was added to each pouch and shaken for one minute. After the ASC treatment, green onions and baby spinach samples were rinsed with sterile DI-water for an additional 30 seconds to remove residual chlorine compounds on the produce. The control (washed) green onion and baby spinach samples were washed for 1 minute with DI-water and also rinsed for an additional 30 seconds as in the ASC-treated samples.

For the mild heat treatments, samples were placed in sterile plastic pouches and treated with sterile DI-water at 40, 45, or 50°C for 1 minute. Control samples (washed) of green onions and baby spinach leaves were washed with ~20°C sterile DI-water. Unwashed control samples were also included in the study.

Combined treatments of UV, ASC, and mild heat. *E. coli* O157:H7 cocktail inoculated green onion and baby spinach samples were prepared following the procedure described above. The combined decontamination treatment was applied with the selected UV treatment first, followed by the selected ASC treatment at the selected temperatures.

Pathogen enumeration. Pathogen enumeration was performed on 20 g green onion and 10 g baby spinach samples immediately after each treatment. Green onion and baby spinach samples were diluted with 180 and 90 ml sterile 0.1% peptone water (1:10, w/v), respectively. The samples were stomached for two minutes using a Seward 400 stomacher (Brinkmann, Westbury, NY). Appropriate dilutions were plated in duplicate on the surface of Violet Red Bile Agar (VRBA) supplemented with 4-methylumbelliferyl- β -D-glucuronide (MUG) for *E. coli* O157:H7 identification. VRBA-MUG plates were incubated at 37°C for 24 h, and *E. coli* O157:H7 colonies were identified by enumerating typical red colonies containing a zone of precipitation and production of blue fluorescence when exposed to long-wave UV light (366 nm). The limit of detection was 10 CFU/ml.

Statistical analysis. All experiments were replicated at least three times, with three samples per replication. The statistical analysis was performed using JMP statistical software (version 9.0, 2010, SAS Institute, Cary, N.C.). One-way ANOVA and

Tukey's one-way multiple comparisons were used to evaluate significant effects of the decontamination treatments on the bacterial populations. Censored data was not included in the statistical analysis and data are presented as < 1 log (detection limit). The significance level was set at $P < 0.05$.

RESULTS

Effects of UV treatment on spot and dip-inoculated green onions. Spot-inoculated green onions had an average initial contamination level of 7.0 log CFU/spot *E. coli* O157:H7 43895 (Figure 2.1A). The pathogen population was significantly decreased by 1.9 log CFU/spot after a UV application of 12.5 mJ/cm². An increased dose of UV (125 mJ/cm²) yielded a 2.6 log reduction in *E. coli* O157:H7 populations, which was significantly higher than the 12.5 and 25 mJ/cm² treatments (Figure 2.1A). The highest level UV dose treatment (500 mJ/cm²) resulted in a 2.8 log average reduction compared to the control, but there was no significant difference between the 62.5 and 500 mJ/cm² UV treatments on spot-inoculated green onions (Figure 2.1A).

Dip-inoculated green onions had an average initial contamination level of 6.4 log CFU/g *E. coli* O157:H7 43895. The highest UV exposure treatment (500 mJ/cm²) resulted in an average 1.1 log CFU/g reduction ($P < 0.05$); however, the lower UV doses did not result in significant reductions in the *E. coli* O157:H7 population (Figure 2.1B).

Effects of washing and acidified sodium chlorite (ASC) treatments on spot and dip-inoculated green onions. Washing of spot-inoculated green onions with sterile DI-water for one minute was significantly effective in reducing *E. coli* O157:H7 43895 population by 1.9 log CFU/spot ($P < 0.05$).

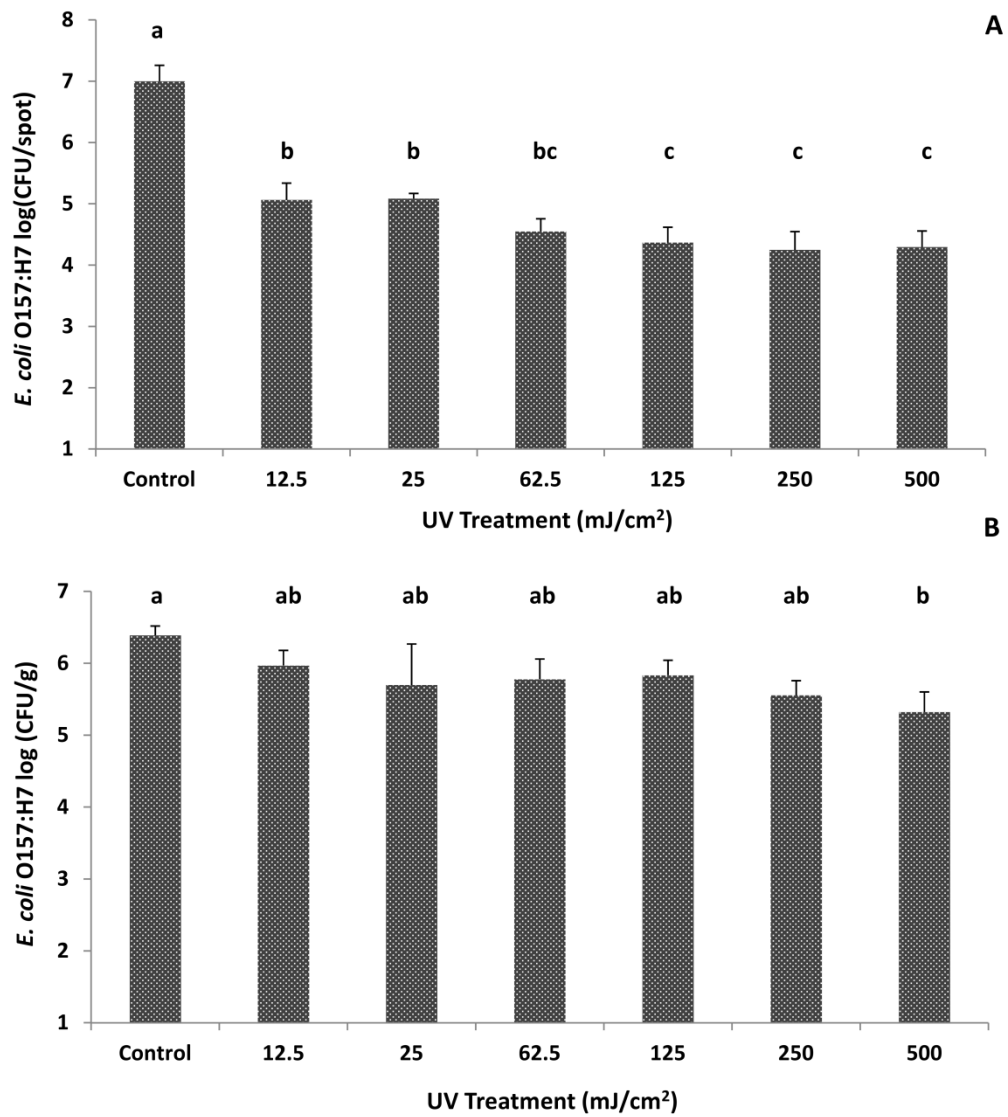


Figure 2.1. Effects of UV treatments on *E. coli* O157:H7 strain 43895 spot (2.1A) and dip-inoculated (2.1B) green onions. Error bars represents the mean \pm SD of counts from three independent replicates, each replicate consisting of at least triplicate observations (n=9). The same letter indicates no significant difference at the 95% confidence level.

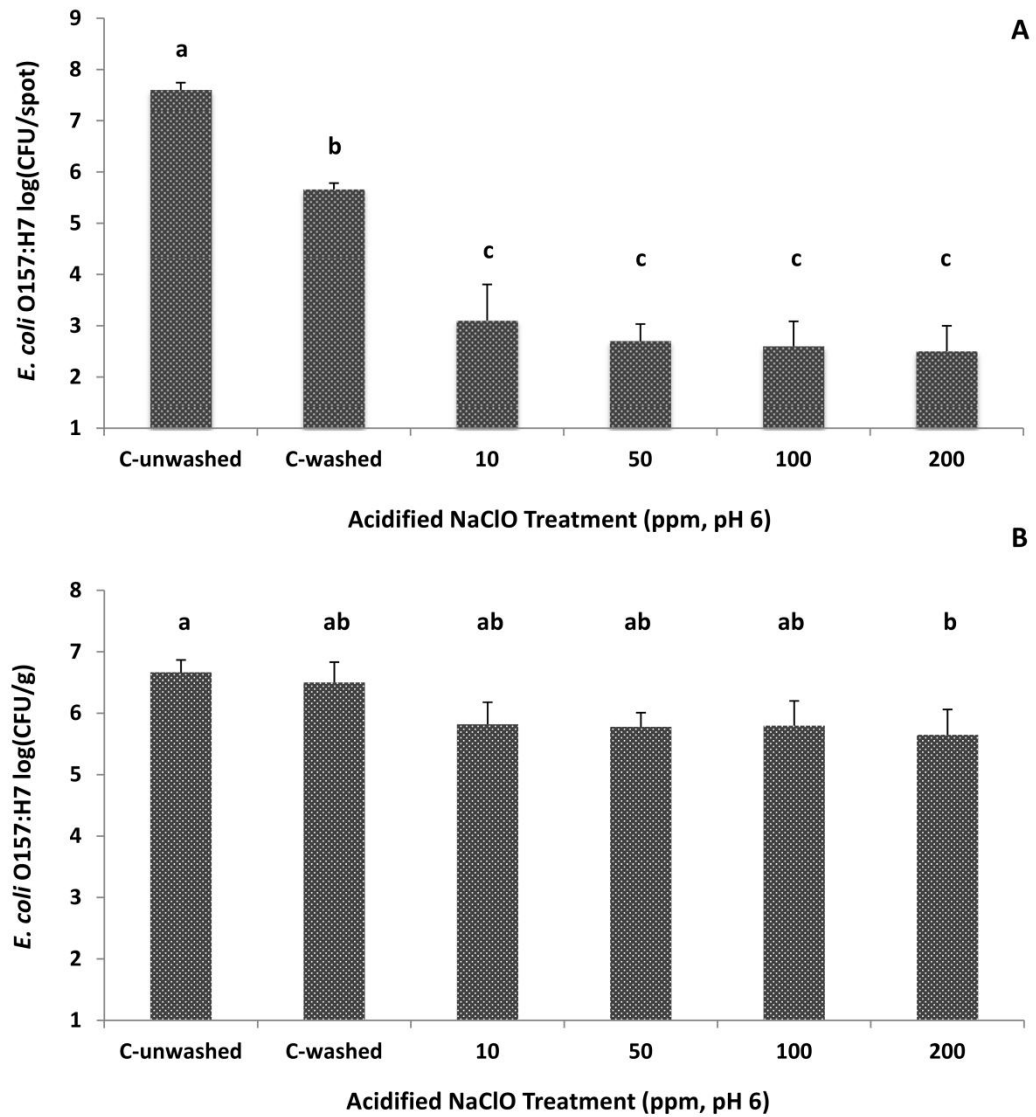


Figure 2.2. Effects of washing and ASC treatments on *E. coli* O157:H7 strain 43895 spot (2.2A) and dip-inoculated (2.2B) green onions. Error bars represents the mean \pm SD of counts from three independent replicates, each replicate consisting of at least triplicate observations (n=9). The same letter indicates no significant difference at the 95% confidence level.

The lowest concentration of ASC treatment (10 ppm) provided an additional 2.6 log CFU/spot reduction compared to the green onions washed with sterile DI-water (Figure 2.2A). However, higher concentrations of ASC (50, 100, 200 ppm) did not significantly increase the efficacy of ASC treatment. The 200 ppm ASC treatment achieved a 3.2 and 5.1 log CFU/spot reduction in *E. coli* O157:H7 43895 populations on green onions compared to the washed and unwashed control green onions, respectively (Figure 2.2A).

However, for dip-inoculated green onions, neither the wash nor the ASC (10, 50, 100 ppm) treatments were statistically significant for the pathogen reduction. The DI-wash treatment reduced *E. coli* O157:H7 populations only by 0.2 log CFU/g. Only the highest concentration of ASC treatment (200 ppm) resulted in significant 1.0 and 0.8 log CFU/g *E. coli* O157:H7 reductions on unwashed and washed green onions, respectively ($P < 0.05$, Figure 2.2B).

Effects of mild heat wash treatments on spot and dip-inoculated green onions.

There were no significant differences among the control (20°C) and 40, 45, and 50°C DI-water wash treatments on spot inoculated green onions ($P < 0.05$). The control wash treatment (20°C) reduced the *E. coli* O157:H7 43895 population by 1.9 log CFU/spot, and the 50°C wash treatment reduced the pathogen population by 2.4 log CFU/spot (Figure 2.3A). On dip-inoculated green onions, the control wash (20°C) and mild heat wash treatments (40, 45, 50°C) were not significantly effective in reducing the *E. coli* O157:H7 population ($P < 0.05$, Figure 2.3B).

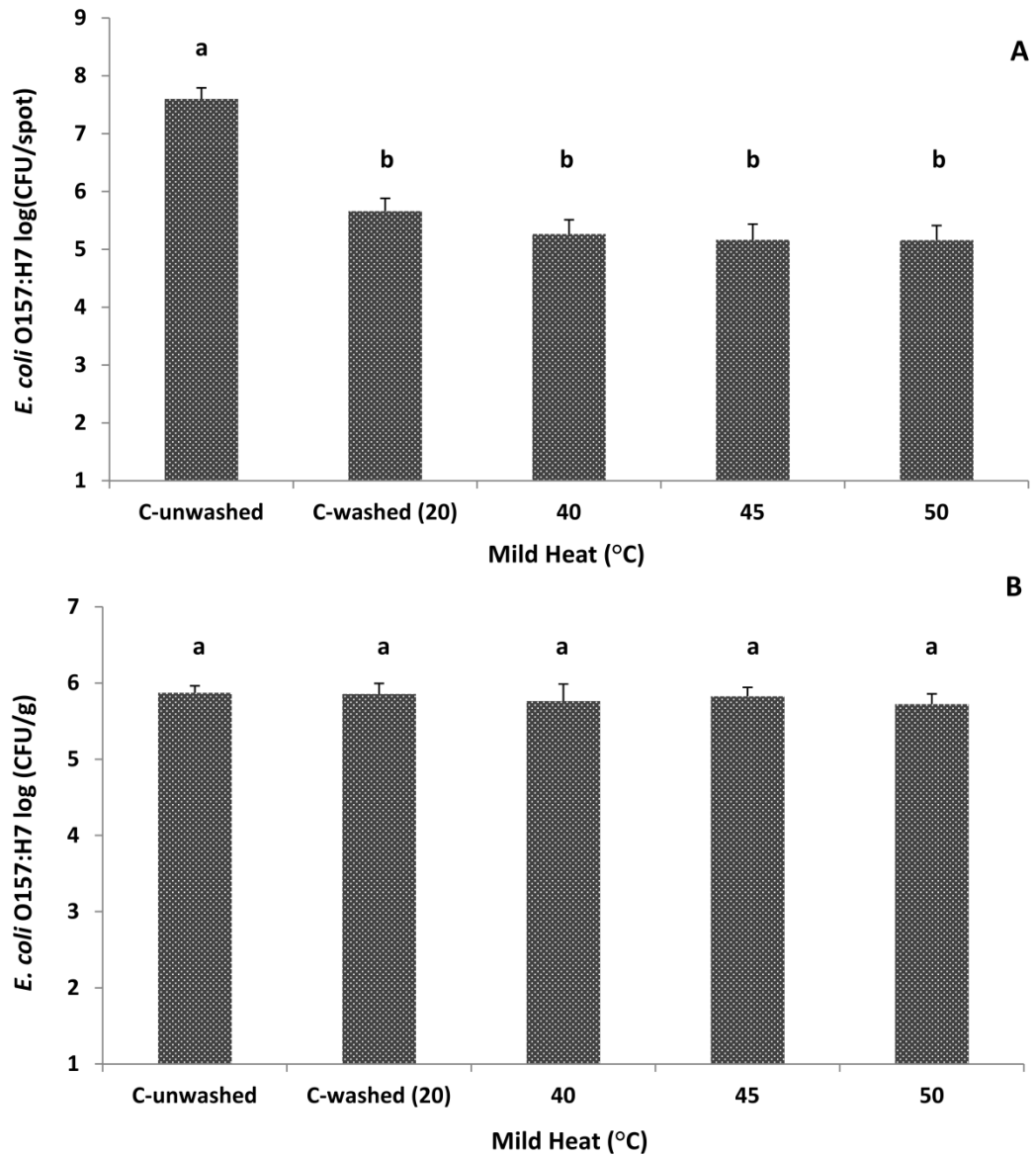


Figure 2.3. Effects of washing and mild heat treatments on *E. coli* O157:H7 strain 43895 spot (2.3A) and dip-inoculated (2.3B) green onions. Error bars represents the mean \pm SD of counts from three independent replicates, each replicate consisting of at least triplicate observations (n=9). The same letter indicates no significant difference at the 95% confidence level.

Effects of UV treatment on spot and dip-inoculated baby spinach. The initial contamination level of *E. coli* O157:H7 43895 on spot-inoculated baby spinach was 6.8 log CFU/spot. A UV treatment of 12.5 mJ/cm² was significantly effective in reducing *E. coli* O157:H7 populations by 1.4 log CFU/spot ($P < 0.05$). Higher doses resulted in a 1.8 log average reduction for UV treatments from 62.5 to 500 mJ/cm², but there was no significant difference between the lowest level (12.5 mJ/cm²) and the other UV exposure levels tested (Figure 2.4A). On dip-inoculated baby spinach samples, UV doses less than 62.5 mJ/cm² did not result in significant pathogen reduction ($P < 0.05$). However, the 62.5 mJ/cm² UV exposure reduced *E. coli* O157:H7 populations by 1.0 log, while a 500 mJ/cm² UV exposure resulted in 1.5 log CFU/g reduction (Figure 2.4B). There was no significant difference in *E. coli* O157:H7 reductions for UV dose levels between 62.5 to 500 mJ/cm² ($P < 0.05$).

Effect of washing and acidified sodium chlorite (ASC) treatments on spot and dip-inoculated baby spinach. Washing spot-inoculated baby spinach leaves with DI-water significantly reduced the *E. coli* O157:H7 43895 populations by 1.0 log on baby spinach ($P < 0.05$). However, there was no significant difference between DI-washed baby spinach samples and ASC-treated samples (Figure 2.5A). The difference between samples that were only washed with DI-water and samples that were treated with 200 ppm ASC samples was marginally insignificant ($p=0.0501$).

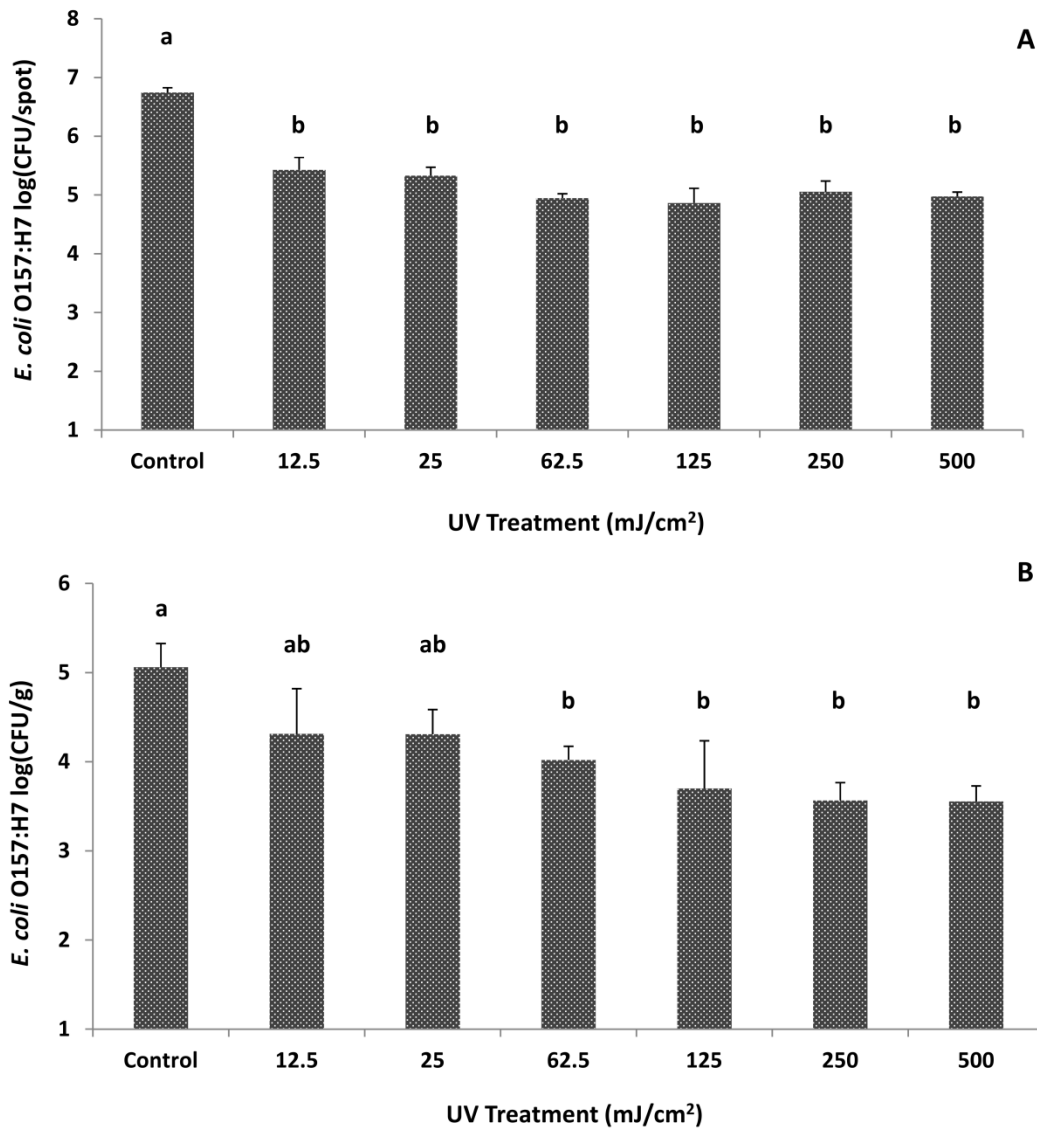


Figure 2.4. Effects of UV treatments on *E. coli* O157:H7 strain 43895 spot (2.4A) and dip-inoculated (2.4B) baby spinach. Error bars represents the mean \pm SD of counts from three independent replicates, each replicate consisting of at least triplicate observations (n=9). The same letter indicates no significant difference at the 95% confidence level.

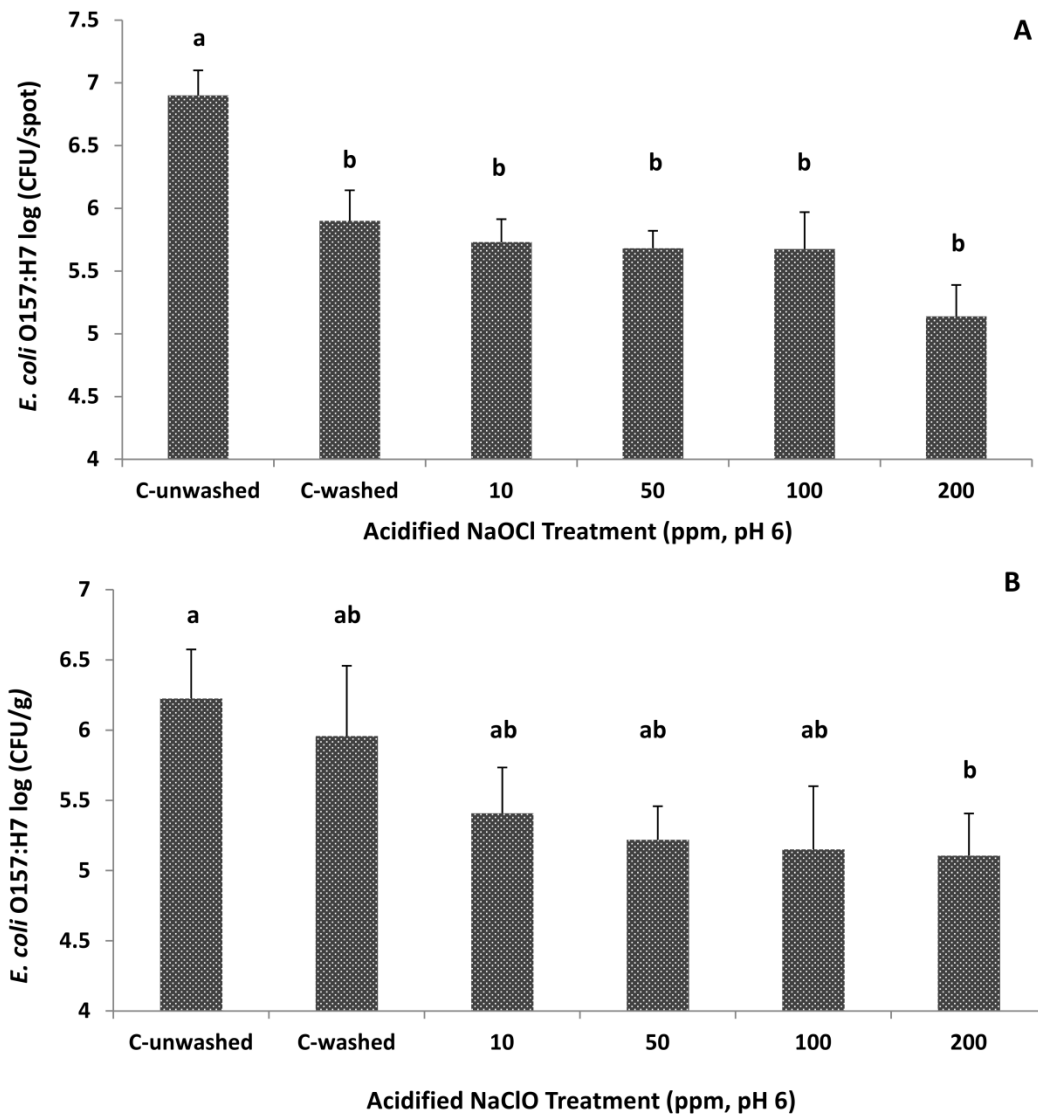


Figure 2.5. Effects of washing and ASC treatments on *E. coli* O157:H7 strain 43895 spot (2.5A) and dip-inoculated (2.5B) baby spinach. Error bars represents the mean \pm SD of counts from three independent replicates, each replicate consisting of at least triplicate observations (n=9). The same letter indicates no significant difference at the 95% confidence level.

Like the spot-inoculated samples, the only significant difference in the dip-inoculated samples was observed between the 200 ppm ASC treated and the unwashed baby spinach samples ($p=0.047$). The 200 ppm ASC treatment resulted in 1.1 log and 0.9 log CFU/g reductions in the *E. coli* O157:H7 43895 populations compared to unwashed and DI-washed control baby spinach samples, respectively. There was no significant difference in the reduction of *E. coli* O157:H7 population between the ASC-treated and control (washed) dip-inoculated baby spinach samples (Figure 2.5B).

Effect of mild heat wash treatment on spot and dip-inoculated baby spinach. For both spot and dip-inoculated baby spinach samples, the efficacy of the mild heat wash treatments (40, 45, 50°C) was not significantly different than the control (20°C) DI-washed samples (Figures 2.6A and 2.6B). However, the washing treatments at all temperatures were significantly effective in reducing *E. coli* O157:H7 43895 populations on spot-inoculated baby spinach samples, but they were all significantly ineffective for reducing the pathogen population on dip-inoculated baby spinach samples ($P < 0.05$).

Effects of combined application of UV, ASC, and mild heat on the inactivation of *E. coli* O157:H7 cocktail contaminated green onions. Since UV treatment at 125 mJ/cm² was significantly effective in reducing *E. coli* O157:H7 43895 populations on spot-inoculated green onions, it was chosen for use in a combined decontamination treatment of an *E. coli* O157:H7 cocktail with 200 ppm ASC treatment at 50°C. The 50°C mild heat wash treatment did not have a noticeable negative impact on the visual appearance of green onions. Both UV and ASC treatments were found individually to be very effective on spot-inoculated green onions. For this reason, the combined treatment was applied to both high (7.2 log CFU/spot) and low (4.3 log CFU/spot)

concentrations of *E. coli* O157:H7 cocktail contaminated green onions (Table 1). At the higher contamination level, the combined treatment (125 mJ/cm² UV, 200 ppm ASC at 50°C) resulted in a 5.9 log CFU/spot reduction, and at the lower contamination level, it resulted in reductions below the detection limit of 1.0 log CFU/spot without a noticeable change in the visual appearance of green onions. The efficacy of the combined treatment was significantly different than the 125 mJ/cm² dose UV treatment alone but not significantly different from the ASC treatments applied at 20°C and 50°C on high level spot-inoculated green onions samples (Table 2.1).

Green onions were also dip-inoculated with the *E. coli* O157:H7 cocktail at high (6.8 log CFU/g) and low (4.0 log CFU/g) concentrations. Because only a UV treatment of 500 mJ/cm² dose was significantly effective in reducing *E. coli* O157:H7 43895 populations on dip-inoculated green onions, it was chosen for use in the combined decontamination treatment along with a 200 ppm ASC treatment at 50°C. The combined treatment resulted in a 2.4 CFU/g reductions in both high and low level cocktail of *E. coli* O157:H7 dip-inoculated green onions, without causing any noticeable change in their appearance (Table 2.1).

Table 2 1. Effect of UV, ASC, mild heat, and combined treatments on reduction of a cocktail of *E. coli* O157:H7 on spot and dip-inoculated green onions.

Treatments	Green Onions				
	Spot-inoculated (CFU/spot)		Dip-inoculated (CFU/g)		
	High Inoculation	Low Inoculation	High Inoculation	Low Inoculation	
C (unwashed)	7.2 ± 0.3 A	4.3 ± 0.4 A	C (unwashed)	6.8 ± 0.2 A	4.0 ± 0.3 A
C (washed)	5.5 ± 0.4 B	2.2 ± 0.2 B	C (washed)	6.6 ± 0.3 AB	3.9 ± 0.2 AB
200 ppm (20°C)	2.6 ± 0.4 D	1.0 ± 0.7 C	200 ppm (20°C)	5.7 ± 0.3 BC	2.8 ± 0.4 BC
200 ppm (50°C)	2.5 ± 0.6 D	0.9 ± 0.8 C	200 ppm (50°C)	5.5 ± 0.4 C	2.7 ± 0.5 C
125 mJ	4.6 ± 0.3 C	0.5 ± 0.7 C	500 mJ	5.3 ± 0.5 C	2.5 ± 0.4 C
125 mJ +200 ppm (50°C)	1.3 ± 0.9 D	< 1.0	500 mJ+ 200 ppm (50°C)	4.4 ± 0.4 D	1.6 ± 0.5 D

Data represents mean log reduction (CFU/g) ± SD (standard deviations) from three independent replicates, each experiment consisting of three observations (n=9). Values in the same column sharing the same letter are not significantly different ($P > 0.05$). Detection limit was 1.0 log CFU/g.

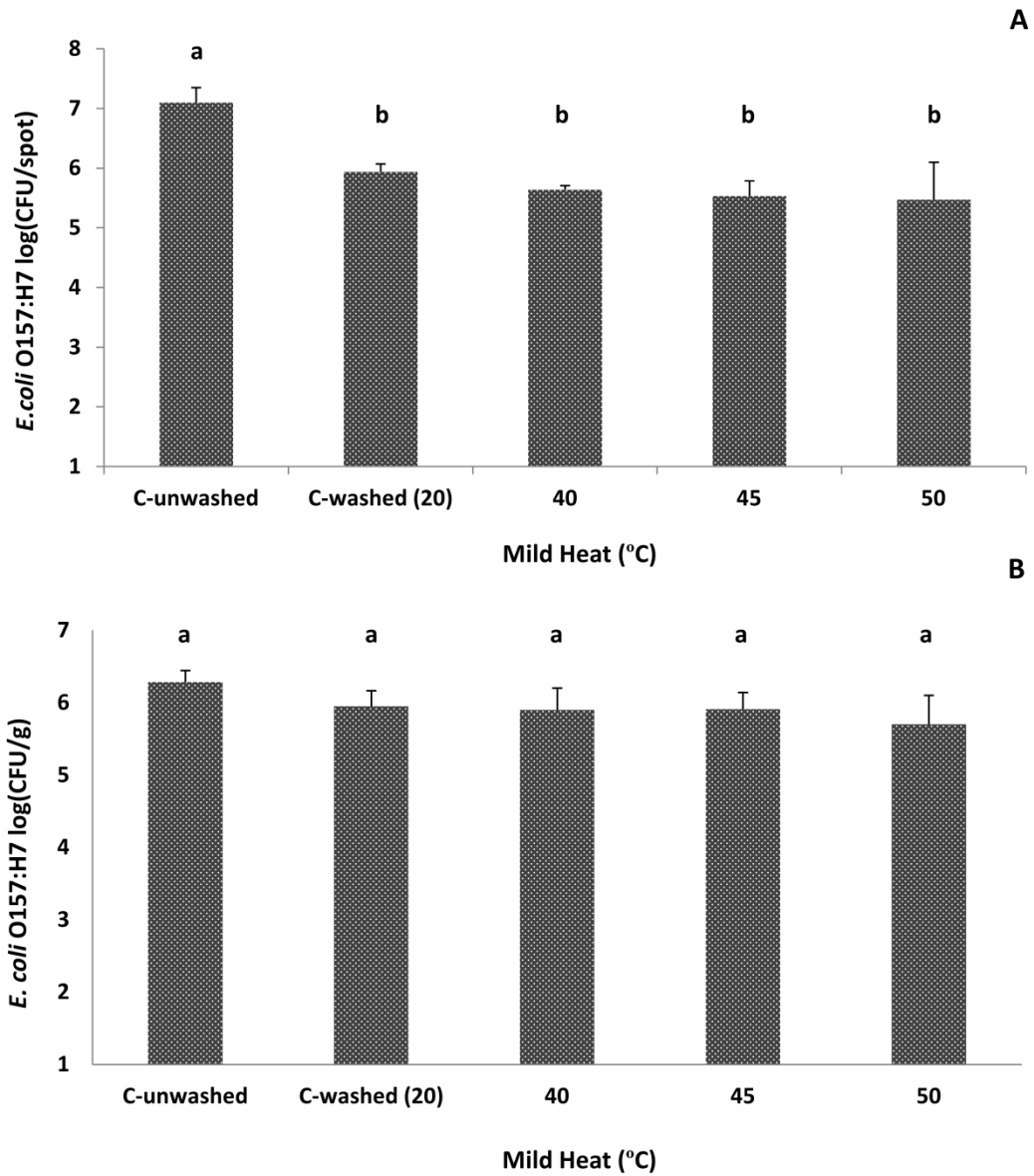


Figure 2.6. Effects of washing and mild heat treatments on *E. coli* O157:H7 strain 43895 spot (2.6A) and dip-inoculated (2.6B) baby spinach. Error bars represents the mean \pm SD of counts from three independent replicates, each replicate consisting of at least triplicate observations (n=9). The same letter indicates no significant difference at the 95% confidence level.

Effect of combined application of UV, ASC, and mild heat on the inactivation of *E. coli* O157:H7 cocktail contaminated baby spinach. A treatment combining 125 mJ/cm² of UV light and 200 ppm ASC was tested on baby spinach samples. A 200 ppm ASC treatment at 20°C or 50°C resulted in a significant decrease in a cocktail of *E. coli* O157:H7 populations both in spot and dip-inoculated control (unwashed) baby spinach samples. However, there was no significant difference between the control (washed) and 200 ppm ASC (20°C) treated spot and dip-inoculated baby spinach samples. On the other hand, while 200 ppm ASC treatment at 50°C was significantly different than control (washed) spot-inoculated baby spinach, the same 200 ppm ASC treatment was not significantly different from the control (washed) dip-inoculated baby spinach samples ($P < 0.05$). Since all the mild heat treatments tested had a negative impact on the visual appearance of baby spinach leaves, determined by informal evaluation by three or four untrained panelists, the mild heat treatments were not incorporated in combined treatments of UV and ASC for baby spinach samples. The optimized treatment (125 mJ/cm² and 200 ppm ASC) resulted in 2.8 log and 1.7 log reductions compared to unwashed and washed spot-inoculated baby spinach samples, respectively (Table 2.2).

Table 2.2. Effect of UV, ASC, mild heat, and combined treatments on reduction of a cocktail of *E. coli* O157:H7 on spot and dip-inoculated baby spinach.

Treatments	Baby Spinach			
	Spot-inoculated (CFU/spot)		Dip-inoculated (CFU/g)	
C (unwashed)	7.0 ± 0.2	A	5.1 ± 0.3	A
C (washed)	5.9 ± 0.1	B	4.6 ± 0.2	AB
200 ppm (20°C)	5.1 ± 0.2	BC	3.9 ± 0.3	BC
200 ppm (50°C)	4.6 ± 0.4	CD	3.7 ± 0.4	BC
125 mJ	5.4 ± 0.3	BC	3.6 ± 0.4	C
125 mJ+200 ppm	4.2 ± 0.5	D	2.5 ± 0.3	D

Data represents mean log reduction (CFU/g) ± SD (standard deviations) from three independent replicates, each experiment consisting of three observations (n=9). Values in the same column sharing the same letter are not significantly different ($P > 0.05$). Detection limit was 1.0 log CFU/g.

Similar results were observed with dip-inoculated baby spinach samples, but no significant difference was observed between control (washed) and 200 ppm ASC treatments at 20°C and 50°C. Combined treatment with 125 mJ/cm² UV exposure and 200 ppm ASC treatments at 50°C resulted in 2.6 and 2.1 log CFU/g/reduction in dip-inoculated samples ($P < 0.05$). The combined treatment was found to be significantly more effective than treatment with 200 ppm ASC or 125 mJ/cm² UV alone ($P < 0.05$), without noticeably affecting the visual appearance of baby spinach samples.

DISCUSSION

Spot-inoculation mimics the surface contamination of produce by food handlers or surface contact with contaminated irrigation and wash water, soil, manure, and animal feces, whereas the dip-inoculation method was used to model infiltrated pathogens entering through cut, wounded, or injured areas, or from roots and stomata at pre-

harvest and post-harvest points.

The wash treatment using deionized water was significantly effective in reducing the *E. coli* O157:H7 population on spot-inoculated green onions and baby spinach where the treatment most likely removed pathogens on the surface which may not have successfully attached. Although capable of removing unattached pathogens from produce, the wash treatment was not significantly effective in reducing *E. coli* O157:H7 populations on dip-inoculated green onions and baby spinach samples likely to harbor infiltrated and surface attached pathogens.

Acidified sodium chlorite (ASC) treatment was the most effective individual *E. coli* O157:H7 decontamination treatment for spot-inoculated green onions. However, it should be noted that while 10 ppm ASC resulted in 4.5 log reductions of *Escherichia coli* O157:H7 populations, 200 ppm ASC treatment resulted in a 5.1 log reduction on unwashed spot-inoculated green onions, and there were no significant differences among the efficacies of ASC treatments (Figure 2.2A). Thus, this finding indicates that if the pathogen is accessible on the surface of green onions, even low level (10 ppm) ASC treatment is effective for significant inactivation of the *E. coli* O157:H7 population, and increased concentration of ASC (free chlorine) will not enhance its efficacy for inaccessible pathogens. On the other hand, ASC (10, 50, 100 ppm) and DI-wash treatments were not significantly effective for reducing the *E. coli* O157:H7 population on dip-inoculated green onions where the pathogens were able to infiltrate into the plant tissue and were inaccessible to ASC. However, a 200 ppm ASC treatment was capable of reducing the population only by 1.0 and 1.1 log CFU/g on dip-inoculated control (unwashed) green onions and baby spinach samples. Similar previous findings of a maximum 1.3 log CFU/g reduction on dip-inoculated baby

spinach was reported with the treatment of 300 ppm sodium hypochlorite (28). Chlorination of lettuce at 200 ppm followed by irradiation at 0.55 kGy resulted in a 5.4-log reduction in *E. coli* O157:H7 levels. Chlorine treatment alone was able to reduce the counts by < 2 logs (15). Even though both UV and ASC treatments were more effective on spot-inoculated samples, they were individually not sufficient for complete inactivation of *E. coli* O157:H7 on green onions and baby spinach. However, a single treatment inactivation efficacy was the greatest for 200 ppm ASC treated spot-inoculated green onions.

It should be noted that the ASC treatments were not significantly different than the DI-wash treatment alone on spot or dip-inoculated baby spinach samples. This result was consistent with the findings of Beuchat *et al.* (1999), which found 200 ppm chlorine and deionized water alone to be equally effective on lettuce (3). Once pathogens are attached, infiltrated, and/or localized in protected areas such as stomata and within the tissue, washing with DI-water and chlorine treatments may have no or very little effect on the removal of foodborne pathogens (37). Similar results have also been reported for lactic acid, phytic acid, calcinated seashells, and chitosan, which showed no significantly higher pathogen reduction than produce washed with only water (19).

UV light (UV-C: 200-280 nm, and UV-B: 280-320 nm) has a germicidal effect on wide range of microorganisms through inactivation via DNA damage and prevents microbial growth and subsequent potential infection. The decontamination of fresh produce using germicidal UV light is an alternative surface decontamination method widely used for inactivation of bacteria and viruses in food and pharmaceutical applications. UV treatment has many advantages, including no legal restrictions, does

not leave any residues, and no sophisticated application and safety equipment are required (41). UV light is a non-thermal processing treatment that is currently recognized for use in food treatment which has also been utilized for the inactivation of foodborne pathogens (such as *E. coli* O157:H7) in several types of produce (18, 41). Studies have shown that UV treatment is also effective for inactivating viral pathogens on fresh produce. The effectiveness of UV light on bacteria is positively correlated to the thymine content of its DNA (24). The UV sensitivities of Gram negative pathogens such as *E. coli* O157:H7 and *Salmonella* spp. is much greater than most Gram positive bacteria such as *L. monocytogenes* (31). The UV-C radiation doses required for a 90% reduction (d_R) value of *E. coli* O157:H7 and *L. monocytogenes* on ready-to-eat salad were determined to be 0.21 and 2.48 J/m², respectively (7).

UV treatment demonstrated a higher germicidal effect on the spot-inoculated green onions than the spot-inoculated baby spinach samples. The differences in the decontamination efficacy of UV and ASC treatments on the surface of green onions and baby spinach leaves are likely due to their surface topographies. The surface inoculated *E. coli* O157:H7 was likely sheltered and protected from the germicidal effects of UV and ASC treatments on baby spinach. However, green onions have a smoother surface and possess mucus-like compounds which may interfere with the surface attachment and/or sheltering the pathogen from UV and ASC treatments. On the other hand, a much higher (500 mJ/cm²) UV exposure was needed to achieve the same level of reduction on dip-inoculated green onions than dip-inoculated baby spinach (62.5 mJ/cm²). This difference was more likely due to a higher infiltration of *E. coli* O157:H7 into green onions than baby spinach as well as pathogens located in protected areas and in the curves of green onions.

Green onion-related foodborne outbreaks have been mostly associated with surface contamination due to poor hygiene practices during the food handling (touch) by infected workers (1). In 2003, green onions were implicated as the source of a Hepatitis A virus (HAV) outbreak resulting in 555 cases and 3 deaths (4). Also in 2003, two HAV outbreaks were linked to green onion consumption from single restaurants in Ohio (9) and Pennsylvania (40). Thus, a successful surface decontamination of green onions could prevent potential foodborne related outbreaks.

The selected UV, ASC and mild heat treatments were used in combination treatments for the decontamination of spot and dip-inoculated green onions and baby spinach. ASC (200ppm) was used in the combined treatments, although the difference between the 10 ppm and 200 ppm treatments was not significant, because the efficacy of chlorinated water is highly dependent on the concentration of available free chlorine, pH, temperature, organic matter, type of produce and vegetable tissue components (2). During the washing process of fresh produce, a large quantity of organic matter is expected to be released into the wash water, which will rapidly deplete the free chlorine. In addition, the application of chlorine at higher temperatures resulted in a higher germicidal activity but also decreased the amount of chlorine present in the solution (17). For this reason, 200 ppm treatment was used at 50°C in combination with 125 mJ/cm² UV treatment. The combined application of 200 ppm sodium hypochlorite and mild heat treatment (50°C) was found to be effective in reducing *E. coli* O157:H7 populations by 1.2 log and was an effective preservation method for cut lettuce (22).

Since the selected combined treatments of UV, ASC, and mild heat were effective on spot-inoculated green onions, the cocktail of five *E. coli* O157:H7 strains was subsequently tested at two different concentrations. The combined treatment (125 mJ/cm² UV and 200 ppm ASC at 50°C) was capable of reducing the pathogen levels below the detection limit (10 CFU/ml) on low-level spot inoculated green onions. Since an enrichment process was not applied, the combined treatment cannot be credited with the complete inactivation of pathogens. However, the selected combined treatments were significantly effective in reducing the pathogen populations on the produce surface. Researchers have reported that chlorine and other surface effective sanitizers will not be able to completely inactivate *E. coli* O157:H7 in vegetables since pathogens can be protected from the germicidal effects of surface sanitizers when they are located in stomata or penetrate into the inner portions of the leaf through cut or damaged areas. Seo and Frank (1999) reported that many live *E. coli* O157:H7 cells were found in stomata and cut edges following treatment with 20 ppm chlorine (32). Another study showed that *E. coli* O157:H7 cells were protected in damaged lettuce tissues and stomata after treatment with 200 ppm chlorine solution, whereas the majority of cells on the surface were inactivated (38). The main benefit of using chlorinated water is the prevention of cross contamination with pathogens during the produce washing step. However, Lopez-Galvez (2010) reported that sodium hypochlorite (100 ppm) and chlorine dioxide (3 mg/L) are ineffective for the prevention of cross-contamination with *E. coli* cells on fresh-cut lettuce using scanning electron microscopy (SEM), which showed that bacterial cells are mainly located in clusters or tissue stomata (25).

Even though the mild heat treatments tested were not significantly effective for *E. coli* O157:H7 inactivation on green onions and baby spinach when deployed alone, a 200

ppm ASC treatment at 50°C showed enhanced inactivation efficacy on dip-inoculated green onions compared to control (washed) samples. For this reason, ASC treatment was applied at 50°C in the combined treatments of green onions. Mild or hot heat treatment has been used in combination with other methods for the reduction of human pathogens and native microbiota in various produce commodities where the pathogens are infiltrated and surface sanitization methods are insufficient (23). Fen *et al.* (2008) submerged whole cantaloupes into water using the following conditions: 10°C water for 20 min (control), 20 ppm chlorine at 10°C for 20 min, and 76°C water for 3 min. The treatment with hot water was shown to significantly reduce both total plate count (TPC) and yeast and mold counts on the rinds of whole fruits, while neither chlorine nor cold water wash resulted in a significant reduction of microbial populations without affecting the quality of cantaloupes (14). Rahman *et al.* (2011) treated *E. coli* O157:H7 and *L. monocytogenes*-contaminated shredded carrots by dipping into alkaline and acidic electrolyzed water, citric acid, and mild heat. When the dipping temperature increased from 1°C to 50°C, the reduction of total bacteria, yeast and fungi increased significantly. The combination of 1% citric acid and acidic electrolyzed water treatment at 50°C reduced the total bacterial counts and as well as yeast and mold by ~3.7 log CFU/g, and it was effective for the reduction of *L. monocytogenes* populations by 3.97 log CFU/g and *E. coli* O157:H7 populations by 4 log CFU/g (30). However, the adverse effects of mild heat treatments on the visual appearance of baby spinach have limited their use in the combined applications.

CONCLUSIONS

Good agricultural practices (GAP), good manufacturing practices (GMP), good handling practices (GHP), Hazard Analysis Critical Control Points (HACCP) programs and other farm-to-fork guidelines for general and produce-specific recommendations can help prevent pathogens introduction and infiltration into produce. Our results indicate that chlorine or another single treatment may not be suitable for the decontamination of all types of fresh produce. Adoption of the optimized treatment combining UV, ASC, and mild heat could enhance the safety of fresh spinach and green onions. The surface decontamination methods tested were more effective on green onions than on baby spinach samples. Because the germicidal efficacy of surface decontamination methods varies with the type of produce, characteristics of their surfaces, pathogen-produce interactions, and modes of contamination, intervention methods specific to the produce of interest and pathogen need to be validated and developed. Infiltrated pathogens remain a challenge for existing decontamination methods.

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CHAPTER 3

GASEOUS ETHYL PYRUVATE DECONTAMINATION OF GREEN ONIONS AND BABY SPINACH

ABSTRACT

Foodborne illnesses associated with fresh produce continue to be a major concern as consumers' demand for healthier and non-thermally processed food increases. However, many commercial disinfectants and antimicrobials are not effective against pathogens internalized in plant tissue. The objective of this study was to evaluate gaseous ethyl pyruvate (EP; CAS 617-35-6) as a safe, alternative antimicrobial agent for the decontamination of *Escherichia coli* O157:H7 on green onions and spinach. Baby spinach leaves and green onions were inoculated with a five-strain cocktail of *E. coli* O157:H7 (pGFP) using the dip-inoculation method. Samples were treated with concentrations of 0, 40, 100, and 400 µl/L gaseous ethyl pyruvate in a 2.6 L enclosed container. The efficacy of gaseous EP in reducing the population of *E. coli* O157:H7_(GFP) on green onions and baby spinach at 4°C and 10°C was monitored over the course of 7 and 5 days, respectively. The lowest EP concentration (40 µl/L) resulted in a 1.7 log CFU/g reduction in *E. coli* O157:H7_(GFP) on green onions after 7 days at 4°C and 1.9 log CFU/g reduction after 5 days at 10°C ($P < 0.05$). In baby spinach, the same concentration resulted in 0.9 log CFU/g and 1.4 log CFU/g ($P < 0.05$) of *E. coli* O157:H7_(GFP) in 7 days at 4°C and in 5 days at 10°C, respectively. On green onions, the highest concentration of EP (400 µl/L) reduced the population of *E. coli* O157:H7_(GFP) > 4.7 log CFU/g in 7 days at 4°C, and 5 days at 10°C. The same concentration was also effective in reducing *E. coli* O157:H7_(GFP) populations

in baby spinach by 4.3 log in 7 days at 4°C and > 6.5 log in 3 days at 10°C. While the successful EP treatments minimally affected the sensory attributes of green onions, the treatments resulted in significant changes in the sensory attributes of baby spinach samples stored at 4°C and 10°C. These results indicate that ethyl pyruvate is an effective antimicrobial that could be used to enhance the safety of selected fresh produce.

INTRODUCTION

The consumption of minimally processed and fresh-cut vegetables and fruits is gaining importance with increasing consumer awareness of their potential health benefits (7). As a result, a greater variety of prewashed, ready-to-eat, minimally processed fruits and vegetables has been introduced into the retail market over the past few years to meet consumer demand for healthy, convenient products.

However, foodborne illness associated with fresh produce continues to be a major concern, particularly because of the increasing reliance on imported food and the globalization of the food supply (27). The proportion of foodborne outbreaks attributed to produce has increased dramatically from 0.7% in the 1970s to 6% in the 1990s and 13% between 1990 and 2005 (9, 33). Recent outbreaks of *E. coli* O157:H7 linked to the consumption of contaminated leafy greens has increased consumer awareness of the potential health risks associated with the consumption of fresh leafy greens (1, 8).

While good agricultural practices (GAPs), good manufacturing practices (GMPs), and good handling practices (GHPs) should be applied to reduce the likelihood of contamination of fresh produce, it is not possible to completely guarantee that produce will be protected from the introduction of foodborne pathogens at all stages in the farm-to-fork processing chain. The introduction of foodborne pathogens may occur via direct contact with contaminated soil, water, manure,

feces, and dust, as well as through poor hygiene and handling practices (5). While surface contamination of produce occurs at a higher frequency, recent studies have suggested that *E. coli* O157:H7 can infiltrate the tissues of crops such as lettuce, spinach, and tomato (36, 41). Foodborne pathogens can enter the edible portion of plants through the stomata, root system, seed coat, wounds, or bruises on the surface of a leaf or fruit during pre or post-harvest processing (9, 23). Once pathogens are internalized, they are shielded from surface decontamination methods. To ensure produce safety and reduce the likelihood of outbreaks of produce-related foodborne illness, effective intervention methods need to be developed and deployed.

Many treatments have been proposed for the decontamination of fresh produce, including single or combined application of chemical sanitizers, detergents, organic acids, ultraviolet (UV) light, ozonation, pulsed UV light, high pressure, ionizing irradiation, and several other treatment methods (4, 31, 32, 40, 42, 43). Currently, chlorinated and non-chlorinated chemical disinfectants are the most common disinfection methods used in produce processing plants. However, it has been demonstrated that these methods achieve less than a 2 log reduction in *E. coli* O157:H7 populations on leafy greens (3, 26). The efficacy of chlorinated water is highly dependent on surface accessibility, pH, the concentration of available free chlorine, temperature, duration of treatment, organic matter, produce type (2). In addition, the possible formation of trihalomethanes (THMs) and other potentially mutagenic/carcinogenic byproducts has raised concerns regarding the use of chlorine in food sanitation (19).

The effectiveness of surface decontamination methods including chemical sanitizers is largely dependent on the surface structure or topography of the produce, and they are not effective against pathogens that may be hidden in

protected sites within plant tissue (20). Therefore, surface contamination methods cannot be relied on for the complete elimination of the safety risks associated with fresh produce. Gaseous-phase volatile antimicrobials are an alternative method for inactivating *E. coli* O157:H7 and other pathogens within plant structures that cannot be reached by chemicals in aqueous solutions. Several antimicrobials in the gaseous phase, including chlorine (14), ozone (40), allyl isothiocyanate (28, 29), methyl isothiocyanate (21), and essential oils (13), have been used to inactivate pathogens on fresh produce. While effective, these methods are complicated, expensive, potentially harmful, or compromise the sensory properties of the produce.

The objective of this study was to evaluate the use of ethyl pyruvate (EP; CAS 617-35-6) as an alternative and safe antimicrobial agent for the decontamination of *E. coli* O157:H7 in green onions and spinach at three different EP concentrations (40, 100, and 400 $\mu\text{L/L}$) and two different storage temperatures (4°C and 10°C). EP (21 CFR 172.515) is classified as GRAS (Generally Recognized as Safe) by the FDA and is currently used as a food additive and flavoring agent. Although EP has been reported to be an anti-inflammatory agent and has been demonstrated to improve survival and ameliorate organ dysfunction in a wide variety of preclinical illnesses, such as stroke, severe sepsis, burn injury, acute respiratory distress syndrome (10, 11), its antimicrobial properties against foodborne pathogens and antimicrobial applications in any food products have not been previously reported.

MATERIALS AND METHODS

Bacterial strains. A cocktail of 5 clinical and food-isolated *E. coli* O157:H7 strains (933, raw hamburger isolate; ATCC 35150, clinical isolate; ATCC 43889, clinical isolate; ATCC 43894, clinical isolate; and ATCC 43895, raw hamburger

meat isolate) were used as inocula in this study. These strains were previously engineered in our laboratory to contain a p-GFP plasmid (Clontech Laboratories, Inc., Palo Alto, CA) that confers inducible expression of green fluorescent protein and ampicillin resistance to facilitate easy enumeration and recovery of injured cells and inhibit/prevent the growth of background microbiota. Comparative studies have shown that the behavior of GFP-engineered *E. coli* O157:H7 strains in food is identical to those of the parental strains (39). The strains were maintained at -80°C with 15% glycerol (Fisher Scientific, Pittsburg, PA). When needed, *E. coli* O157:H7 strains were cultured overnight at 37°C in Tryptic Soy Broth (TSB) medium supplemented with ampicillin ($100\text{ }\mu\text{g/mL}$). The inoculum cocktail (1:100 dilution, $\sim 10^7$ CFU/ml) was prepared by mixing 4 mL of each of the 5 strains (20 mL in total) in 2 L of 0.1% peptone water.

Inoculation of green onions and baby spinach. Fresh green onions and spinach were purchased from a local grocery store (Wegmans, Geneva, NY) the day before each experiment. Each green onion was trimmed to a length of 20 cm. Green onions and spinach were washed 3 times with deionized water and dried in a biosafety cabinet for 30 minutes at room temperature. Green onions and baby spinach were then inoculated using standard dip inoculation methods (25) to simulate a worst-case scenario. Specifically, green onion and spinach samples were immersed for 1 minute in a 100-fold diluted overnight *E. coli* O157:H7 culture and then dried in a biosafety cabinet for 2 hours at ambient temperature (20°C).

Application of ethyl pyruvate (EP). Ethyl pyruvate (EP) was applied to *E. coli* O157:H7_(GFP)-contaminated green onions and spinach using a previously published method with slight modifications (28). Briefly, 10 g of inoculated spinach or 40 g of inoculated green onions were placed in sterilized 2.6 L ($29.2\text{ cm} \times 18.7\text{ cm} \times 8.3\text{ cm}$) closed-lid Sterilite® food containers (Townsend, MA). Treatment and control

samples were stored at 4°C for 7 days and at 10°C for 5 days. In addition, a separate study was conducted for EP-treated and control samples placed at 10°C for 12 or 24 hours before cooling to 4°C and storage for up to 5 days. High humidity within each storage container was achieved using a sponge wetted with 20 mL deionized water. To generate EP gas over time, 105 μ L, 260 μ L, or 1040 μ L (equivalent to 40, 100, 400 μ L/L of air) of EP solution (98% purity; Sigma-Aldrich, St. Louis, MO) was deposited onto KimWipes tissues. Direct contact with the spinach or green onions and the sponge and the EP-impregnated KimWipes tissues was avoided even though EP is classified as a GRAS food additive.

Sensory analysis. Sensory analysis was conducted on non-inoculated green onions and baby spinach controls (no EP treatment) and EP-treated samples (40 μ L/L, 100 μ L/L, and 400 μ L/L). Samples stored at 4°C were tested on days 0, 1, 3, and 7, and samples stored at 10°C were tested on days 0, 1, and 3. Samples were visually inspected for color, odor, texture, and overall quality (acceptability) by a semi-trained panel of 20 using a 9-point hedonic scale, where a score of 1 represented “dislike extremely” and a score of 9 represented “like extremely.” The green onion and baby spinach samples were considered acceptable if their mean score for overall acceptability was above 5 (neither like nor dislike). The samples from each treatment (the control and three levels of EP) were served in Sterilite® food containers labeled with a random 3-digit number and all four samples were presented at once. The lids of the food containers were removed 15 minutes before the sensory analysis.

Color analysis. The color of spinach samples was measured using a HunterLab Spectrophotometer (Model UltraScan XE, Reston, VA) using a 9.525 mm measuring aperture. The colorimeter was calibrated using the standard white and black tiles. Hunter *L*, *a*, and *b* values were obtained on both sides of 5 spinach

leaves at 3 random locations and the mean L (light-dark), a (red-green), and, b (yellow-blue) values were calculated. Color analysis was not performed on green onions because the measuring aperture was larger than the green onion leaves.

Gas chromatography-mass spectroscopy (GC-MS) analysis. EP solutions equivalent to 40 $\mu\text{L/L}$, 100 $\mu\text{L/L}$, and 400 $\mu\text{L/L}$ (39.2 $\mu\text{g/L}$, 98 $\mu\text{g/L}$, and 392 $\mu\text{g/L}$, respectively) of air were deposited onto KimWipes (Kimberly-Clark, Roswell, GA) tissues to act as an EP reservoir and were placed in closed-lid containers as described above. The lids of the containers were punctured with a push pin, and a self-adhesive rubber septum was placed over the hole. Headspace samples (5 μL) were sampled using a gas-tight syringe and immediately injected onto Varian CP-3800 gas chromatograph coupled to a Saturn 2000 ion trap mass spectrometer (Varian, Walnut Creek, CA). Injections were splitless, and the injector temperature was 250°C. Separation was achieved on a Varian VF-WAXms 30 m \times 0.25 mm internal diameter (ID), 0.25 μm film thickness column preceded by Restek (Bellafonte, PA) deactivated silica guard column (10 m \times 0.25 mm ID). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. After injection, samples were held at an initial column temperature of 80°C for 5 min; the column temperature was then increased by 20°C/min to 200°C. Calibration standards were prepared with 9.85, 19, 78, 295, and 1121 $\mu\text{g/L}$ EP in dichloromethane solvent. One μL of each liquid standard was injected, and a calibration curve constructed “Peak area” vs “ μg EP injected”). The injector setting for headspace and liquid injections were identical. The quantification was $m/z=43$. Headspace analysis was replicated two independent times. Each replicate was the average of two subsequent injections per sample.

Microbial analysis. EP-treated and control samples of green onions and spinach were pummeled (1:5, w/v) in sterile 0.1% peptone water using a Stomacher Lab-

Blender 400 (Tekmar Company, Cincinnati, OH). Further 10-fold serial dilutions were made using sterile 0.1% peptone water. Appropriate dilutions were plated on Tryptic Soy Agar (TSA) supplemented with ampicillin (100 µg/mL) and 20 µg/mL of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher Scientific, Pittsburg, PA) and incubated at 37°C for 36 to 48 hours to resuscitate injured pathogens. Green fluorescence produced by the surviving pathogens was visualized using a UV light (365 nm) transilluminator for colony counting.

Statistical analysis. A randomized factorial block design that included three concentrations of gaseous EP (40, 100, 400 µL/L air), two temperatures (4°C and 10°C), and six sampling times (0, 0.5, 1, 3, 5, and 7 days) was used. All EP decontamination experiments were replicated three independent times, each with two samples per replication. Statistical analysis was performed using JMP statistical software (version 9.0, 2010, SAS Institute, Cary, NC). One-way ANOVA and Turkey multiple comparison tests were used to compare significant differences in the bacterial populations and sensorial properties of EP-treated and control green onions and baby spinach. Censored data was not included in the statistical analysis, and data are presented as < 0.5 log (detection limit). The significance level was set at $P < 0.05$.

RESULTS

Inactivation of *E. coli* O157:H7_(GFP) on green onions at 4°C and 10°C. The initial *E. coli* O157:H7_(GFP) contamination level on green onions was confirmed as 5.2 log CFU/g by plating on Tryptic Soy Agar (TSA) supplemented with ampicillin (100 µg/mL) and 20 µg/mL of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Figure 3.1A). While the *E. coli* O157:H7_(GFP) population decreased about 1.4 log cycle in the control samples, the lowest concentration of EP (40 µL /L) did not

result in any significant inactivation compared to the control samples over 7 days of storage at 4°C (Figure 3.1A). However, 100 and 400 µL/L concentrations of EP reduced the *E. coli* O157:H7_(GFP) population by 3.4 and >4.7 log, respectively, on day 7 for samples stored at 4°C.

In green onions stored at 10°C, antimicrobial efficacy against *E. coli* O157:H7_(GFP) was achieved more rapidly (Figure 3.1B). In control samples, the *E. coli* O157:H7_(GFP) population was reduced by 0.8 log after 5 days. After five days of storage, the 40 µL/L treatment resulted in only a 1.9 log reduction, but the 100 µL/L and 400 µL/L treatments reduced the *E. coli* O157:H7_(GFP) population by approximately 4.1 and > 4.7 log, respectively (Figure 3.2; Table 3.1).

Inactivation of *E. coli* O157:H7_(GFP) on baby spinach at 4°C and 10°C. The initial contamination level of baby spinach samples were confirmed as 7.0 log CFU/g *E. coli* O157:H7_(GFP) by plating. After storage for 7 days at 4°C, the *E. coli* O157:H7_(GFP) populations were reduced by 0.5 log CFU/g in the control samples and 0.9 log CFU/g in the samples treated with 40 µL/L EP; the difference was not significant ($P \leq 0.05$) (Figure 3.2A; Table 3.1). A significant increase in the efficacy of EP against *E. coli* O157:H7_(GFP) at 4°C was observed at the higher concentrations (Table 3.1). After 7 days of incubation at 4°C, *E. coli* O157:H7_(GFP) populations were significantly reduced by 2.8 log CFU/g in the 100 µL/L EP treatment and by 4.3 log CFU/g in the 400 µL/L EP treatment ($P < 0.05$); Table 3.1).

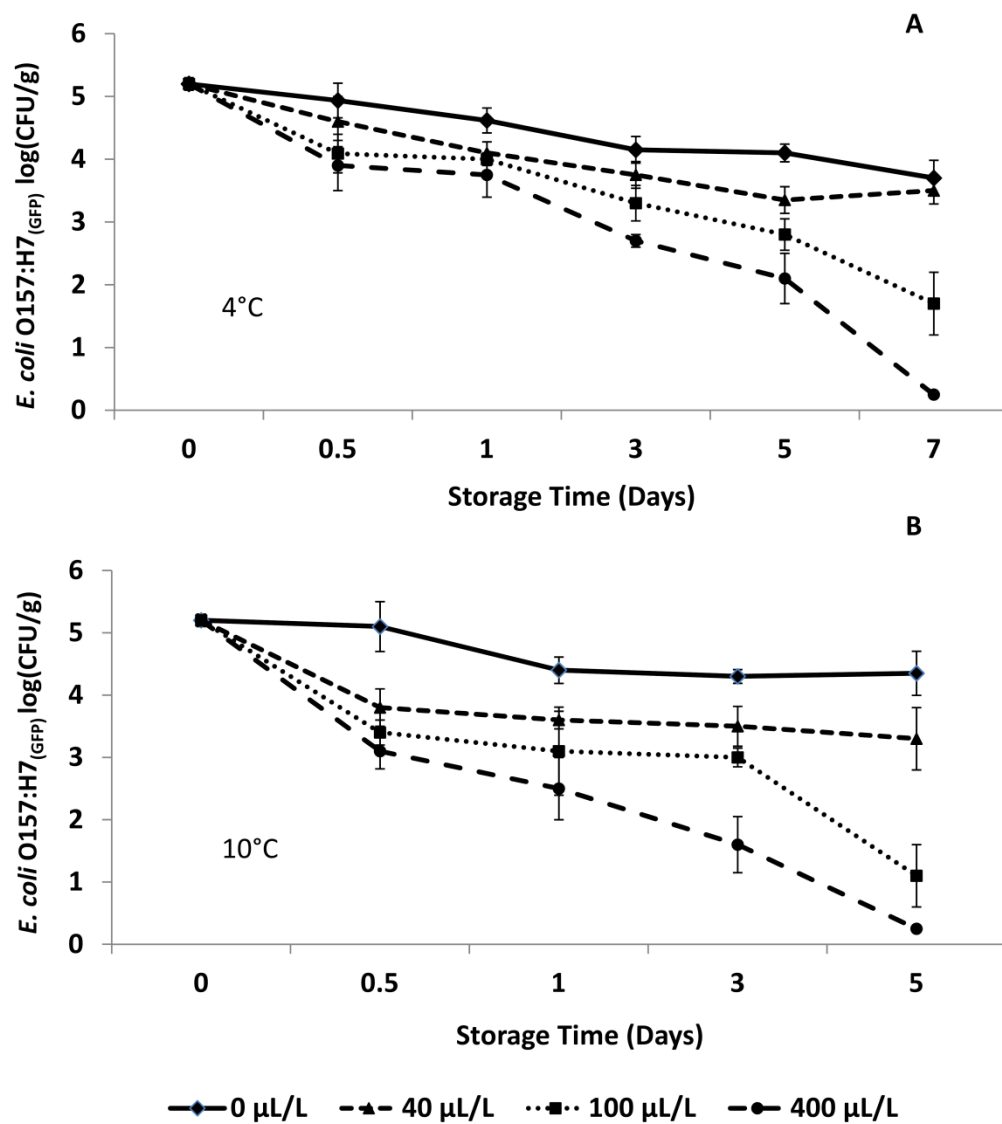


Figure 3.1. Inactivation of *E. coli* O157:H7 on green onions by gaseous (EP) at 4°C (3.1A) in 7 days and 10°C (3.1B) in 5 days. Error bars represents the mean \pm SD of counts from three independent replicates, each replicates consisting of duplicate observations (n=6). Detection limit was 0.5 log CFU/g.

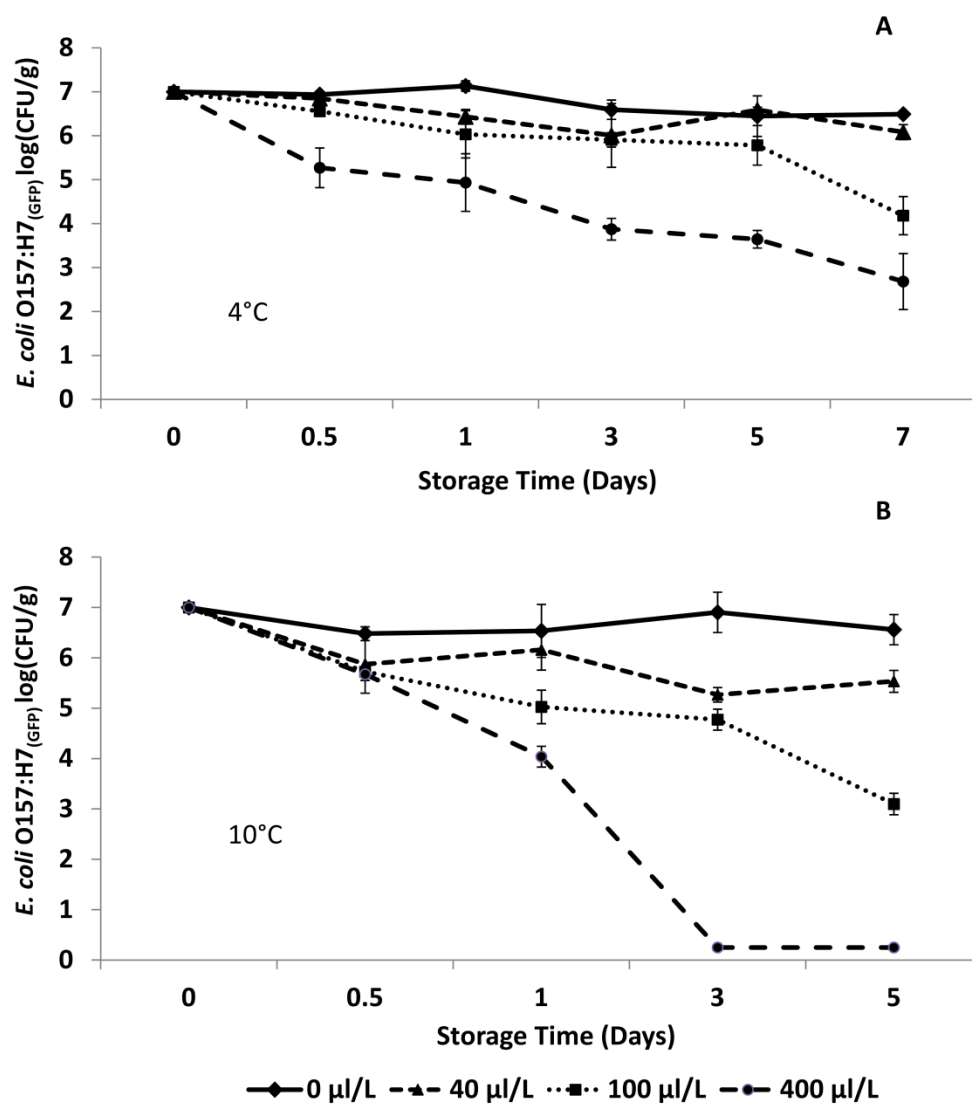


Figure 3.2. Inactivation of *E. coli* O157:H7_(GFP) on baby spinach by various concentrations of gaseous ethyl pyruvate (EP) at 4 °C (3.2A) in 7 days and 10°C (3.2B) in 5 days. Error bars represents the mean \pm SD of counts from three independent replicates, each replicates consisting of duplicate observations (n=6). Detection limit was 0.5 log CFU/g.

As with the green onion samples, the antimicrobial activity of EP against *E. coli* O157:H7_(GFP) on baby spinach was also higher at 10°C. The population of *E. coli* O157:H7_(GFP) was reduced by 0.4 log CFU/g on control samples after 5 days of storage at 10°C (Figure 3.2B). After 5 days at 10°C, EP concentrations of 40 µL/L resulted in a 1.4 log CFU/g reduction of *E. coli* O157:H7_(GFP), and 100 µL/L EP resulted in a 3.9 log CFU/g reduction. The most rapid control occurred at the highest EP concentration: after only 3 days of incubation at 10°C, the 400 µL/L EP treatment resulted in >6.5 log CFU/g reduction of *E. coli* O157:H7_(GFP) (Table 3.1).

Inactivation of *E. coli* O157:H7_(GFP) on green onions and baby spinach by shifting the storage temperature from 10°C to 4°C after 12 h or 24 h. While 400 µL/L gaseous EP was more effective in inactivating *E. coli* O157:H7_(GFP) at 10°C than at 4°C, the sensory properties of baby spinach were negatively affected by the higher storage temperature in both EP-treated and control samples. To test the effect of a shorter exposure to 10°C on the efficacy of EP, samples were stored at 10°C for 12 or 24 hours before cooling to 4°C. In green onions contaminated with 5.8 log CFU/g *E. coli* O157:H7_(GFP) and treated with an initial 12 h storage at 10°C, after 5 days the control samples had a 1.6 log reduction in the *E. coli* O157:H7_(GFP) population, and samples treated with 400 µL/L EP were reduced more than >5.3 log (Figure 3.3A). The same results were obtained for the 400 µL/L gaseous EP treatment stored at 10°C for 24 h followed by 4°C for additional 4 days (Figure 3.3B).

Similarly, a 7.1 log CFU/g *E. coli* O157:H7_(GFP)-contaminated control and 400 µL/L gaseous EP-treated baby spinach samples were stored at 10°C for 12 h (Figure 3.3A) or 24 h (Figure 3.3B).

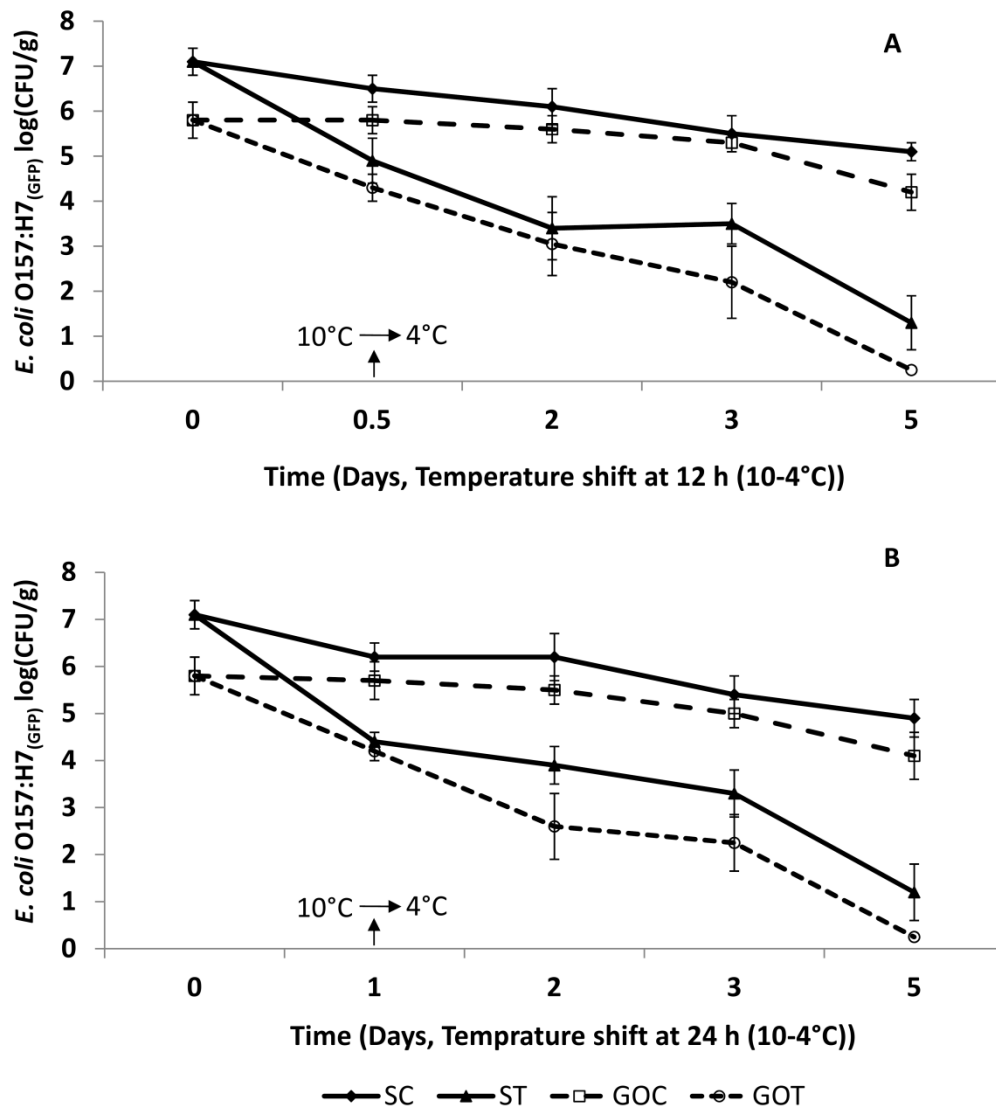


Figure 3.3. Inactivation of *E. coli* O157:H7_(GFP) on baby spinach and green onions by 400 μ L/L gaseous EP with shifting of incubation temperature from 10°C to 4°C after 12 h (3.3A) and after 24 h (3.3B). Error bars represents the mean \pm SD of counts from three independent replicates, each replicates consisting of duplicate observations (n=6). Detection limit was 0.5 log CFU/g. SC, spinach control; ST, spinach treatment; GOC, green onion control; GOT, green onion treatment

Sensory evaluation of green onions and baby spinach. The panelists did not find a significant difference between the color and odor attributes of the control and EP-treated green onions at the end of the 7 day storage period at 4°C (Table 3.3). There were also no significant differences in the textural and overall quality attributes on day 3 for EP-treated samples and control samples stored at 4°C. The length of storage generally had a significant negative effect on all of the sensory characteristics of the green onions (Table 3.3). Although the color attributes of green onions treated with 100 and 400 µL/L EP and stored at 10°C were lower than the control samples, there were no significant differences between EP-treated and control samples for odor and texture properties analyzed on day 1 and day 3. Overall, the panelists generally rated all the sensory attributes of the control and EP-treated green onions as acceptable (sensory scores above 5) over the testing period of 7 days at 4°C and 5 days at 10°C (Table 3.3).

Sensory scores for all EP-treated baby spinach samples (color, texture, overall quality) were generally lower than for the control samples for storage at 4°C and 10°C. When baby spinach leaves were stored at 10°C, even the 40 µL/L EP-treated leaves were significantly different from the control samples. In addition, the texture and overall quality attributes were found to be significantly different for the day 1 and day 3 analyses. The odor ratings of the control and 400 µL/L EP-treated baby spinach samples stored at 4°C for 7 days and 10°C for 3 days were significantly different. For all sensory attributes, storage time resulted in significant differences between the control and treated samples, except odor in the control samples. After 7 days of storage at 4°C, the color attributes of baby spinach treated with the lowest concentration of EP (40 µL/L) were significantly different from the untreated control samples (Table 3.4).

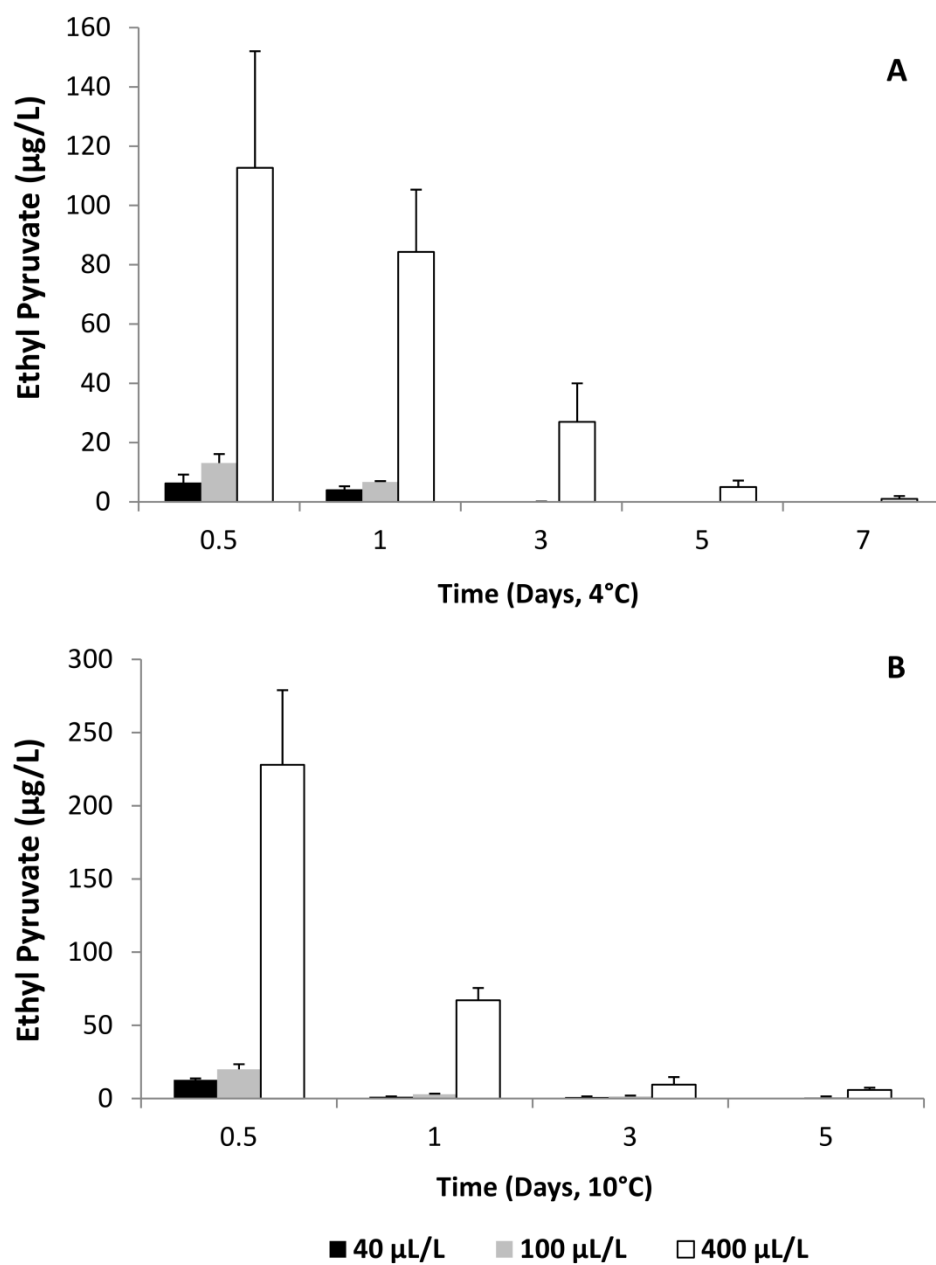


Figure 3.4. Headspace EP concentrations in containers of green onions containing 40, 100, and 400 µL/L EP deposited on KimWipes tissues and stored at 4°C for 7 days (3.4A) and 10°C for 5 days (3.4B). Error bars represents the mean \pm SD of two independent replicates.

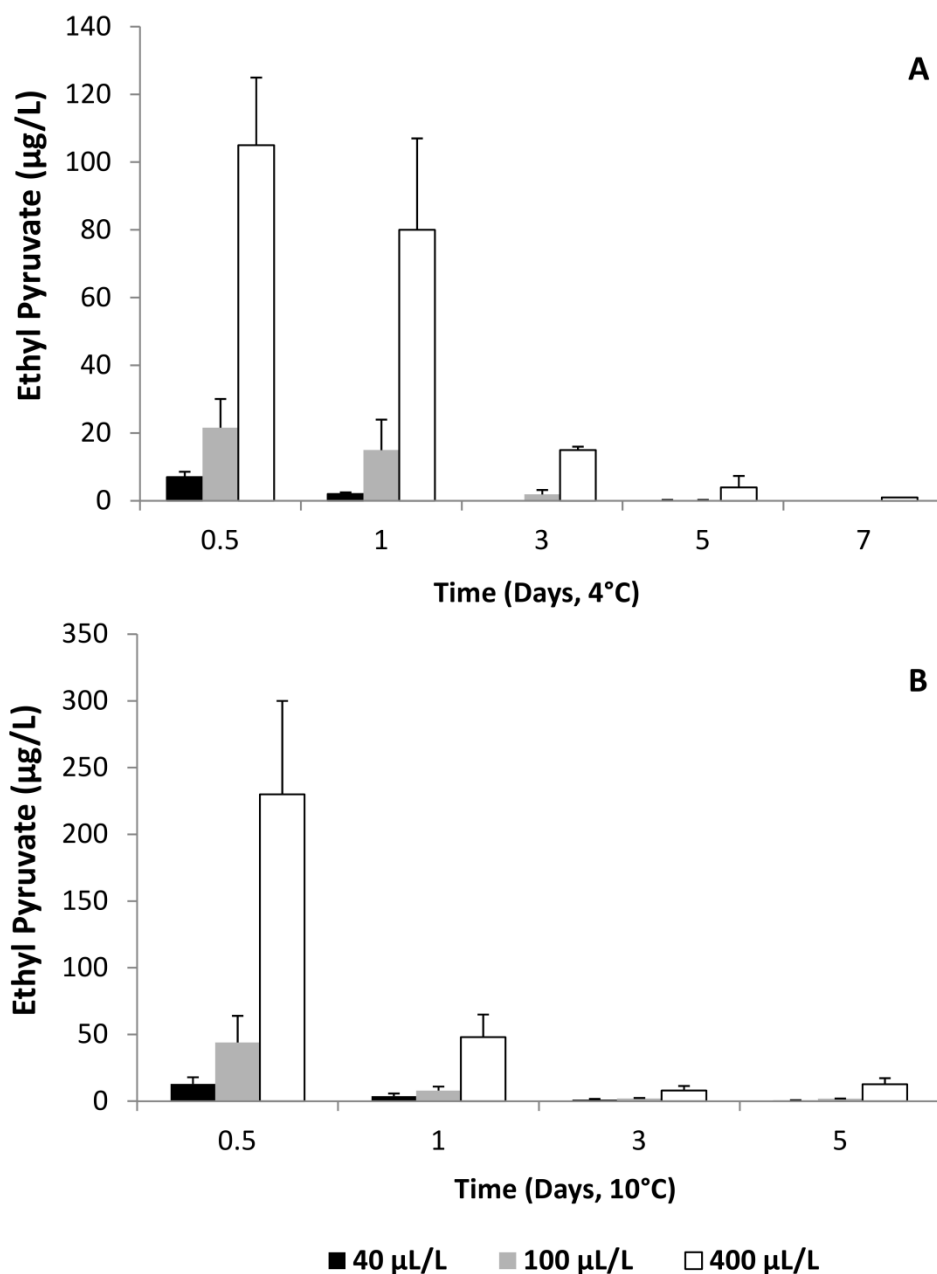


Figure 3.5. Headspace EP concentrations in containers of baby spinach containing 40, 10, and 400 µL/L EP deposited on KimWipes tissues and stored at 4°C for 7 days (3.5A) and 10°C for 5 days (3.5B). Error bars represents the mean \pm SD of two independent replicates.

The samples were then stored at 4°C and the *E. coli* O157:H7_(GFP) population was monitored over 5 days. With the 12 h exposure to 10°C, the *E. coli* O157:H7_(GFP) population of the control and 400 µL/L gaseous EP-treated samples decreased by 2.0 log CFU/g and 5.7 log CFU/g, respectively (Figure 3.3A). In addition, the, 24 h temperature shift analysis showed a 2.0 log reduction in control samples and a 5.9 log CFU/g reduction in 400 µL/L EP-treated samples (Figure 3.3B).

Effect of EP treatment on the color of baby spinach. Color parameter values (*L*, *a*, *b*) were obtained for the upper and lower sides of the spinach leaves. The *L* values increased significantly with increasing storage time in the control and EP-treated samples stored at 4°C and 10°C (Table 3.2). The increase in *L* values reflects the lightening of baby spinach leaves during storage. While *L* values were only significantly different on day 7 and day 3 control samples stored at 10°C and 4°C, respectively, the EP-treated samples generally had significantly higher *L* values at 4°C and 10°C by day 1 compared to day 0 EP-treated samples. On the other hand, the *L* values of the baby spinach leaves did not differ significantly among the control and EP-treated samples on day 7 at 4°C and on day 3 at 10°C (Table 3.2).

The *a* values significantly increased (indicating more redness) with increasing EP concentrations in samples stored at 10°C, whereas in samples stored at 4°C a significant increase in the *a* value indicating loss of green color was only observed for the baby spinach treated with 400 µL/L EP (Table 3.2). There was no significant difference between the *b* values for the upper and lower sides of the spinach leaves at all EP concentrations during 7 days of storage at 4°C and 3 days of storage at 10°C. However, *b* values increased (indicating more yellowness) significantly on the upper side of the spinach leaves with increasing EP concentration and storage time, indicating an increase in yellow color.

Table 3.1. Inactivation of *E. coli* O157:H7_(GFP) on green onions and baby spinach leaves by gaseous EP at 4°C in 7 days and 10°C in 5 days.

Conc (μL/L)/ Day	Reduction (log CFU/g) ± SD*									
	Green Onions					Baby Spinach				
	4°C		10°C			4°C		10°C		
	3	7	1	5		3	7	1	5	
0	0.9 ± 0.2 A	1.4 ± 0.3 A	0.8 ± 0.2 A	0.8 ± 0.3 A		0.4 ± 0.2 A	0.5 ± 0.1 A	0.5 ± 0.2 A	0.4 ± 0.4 A	
40	1.4 ± 0.2 AB	1.7 ± 0.2 A	1.8 ± 0.1 AB	1.9 ± 0.5 A		1.0 ± 0.7 A	0.9 ± 0.2 A	0.8 ± 0.4 AB	1.4 ± 0.2 A	
100	1.9 ± 0.3 BC	3.7 ± 0.6 B	2.2 ± 0.7 B	4.4 ± 0.5 B		1.1 ± 0.2 A	2.8 ± 0.4 B	1.9 ± 0.3 AB	3.9 ± 0.2 B	
400	2.5 ± 0.1 C	>4.7	3.7 ± 0.5 C	>4.7		3.1 ± 0.2 B	4.3 ± 0.6 C	2.9 ± 0.2 B	>6.5	

*Initial contamination level of *E. coli* O157:H7_(GFP) was 5.2 log CFU/g for green onions and 7.0 log CFU/g for baby spinach. Data represents mean log reduction (CFU/g) ± SD from three independent replicates, each experiment consisting of duplicate observations (n=6). Values in the same column sharing the same letter are not significantly different ($P > 0.05$). Detection limit was 0.5 log CFU/g.

The concentration of EP that was most effective in inhibiting *E. coli* O157:H7 (400 μL/L) was associated with a significant difference in color from the control sample after only 1 day of storage at 10°C. Overall, spinach quality was compromised by the 100 μL/L and 400 μL/L EP treatments during storage at 10°C. However, a better preservation of quality properties was observed at 4°C storage. Based on this sensory analysis, all sensory attributes of EP-treated baby spinach were generally found to be unacceptable (sensory score lower than 5) by day 3 of storage at 4°C and by day 1 of storage at 10°C (Table 3.4). The texture properties of control baby spinach samples were also found unacceptable after 3 days of storage at 4°C and 10°C.

Headspace Analysis of EP. Gaseous EP was released into the head space of the storage containers with the green onions or baby spinach, and the concentration of gaseous EP was monitored throughout the study period. After 12 hours of storage, headspace EP concentrations were 112 μg/L for green onion samples stored at 4°C and 228 μg/L for samples stored at 10°C (Figures 3.4A and 3.4B). Maximum EP headspace concentrations in the spinach containers after 12 hours of storage were 105 μg/L for storage at 4°C and 230 μg/L at 10°C (Figure 3.5A and Figure 3.5B).

Table 3.2. Color values of control and EP-treated baby spinach leaves stored at 4°C and 10°C.

<i>Upper Side</i>		4°C				10°C			
Day/Conc (μL/L)		0	40	100	400	0	40	100	400
L*									
0		x(37.5±0.7) ^a	x(37.5±0.7) ^a	x(37.5±0.7) ^a	x(37.5±0.7) ^a	x(37.5±0.7) ^a	x(37.5±0.7) ^a	x(37.5±0.7) ^a	x(37.5±0.7) ^a
1		x(40.8±2.7) ^a	y(41.1±2.3) ^a	y(43.1±0.5) ^a	y(43.3±1.5) ^a	x(39.2±2.3) ^a	x(37.9±1.8) ^a	y(41.1±2.2) ^{a,b}	y(43.3±2.3) ^b
3		x(39.3±1.8) ^a	y(44.0±1.0) ^a	y(41.7±3.7) ^a	y(43.6±1.4) ^a	y(44.5±2.0) ^a	y(46.0±2.3) ^a	z(47.3±2.3) ^a	y(44.6±1.4) ^a
7		y(44.3±1.7) ^a	y(43.0±1.1) ^a	y(44.4±2.5) ^a	y(44.0±1.4) ^a				
a*									
0		x(-9.0±0.6) ^a	x(-9.0±0.6) ^a	x(-9.0±0.6) ^a	x(-9.0±0.6) ^a	x(-9.0±0.6) ^a	x(-9.0±0.6) ^a	x(-9.0±0.6) ^a	x(-9.0±0.6) ^a
1		x(-9.3±0.5) ^a	x(-8.5±1.0) ^a	x(-8.7±1.2) ^a	x(-8.2±1.1) ^a	x _y (-9.5±0.7) ^a	x(-9.7±0.4) ^a	x(-9.7±0.9) ^a	x(-10.2±1.2) ^a
3		x(-8.9±0.5) ^a	x(-8.9±0.3) ^a	x(-7.8±1.2) ^a	x(-7.5±0.3) ^a	y(-10.3±0.3) ^a	y(-7.1±0.9) ^b	y(-6.9±0.2) ^b	y(-3.4±1.8) ^c
7		x(-9.1±0.4) ^a	x(-7.7±0.4) ^a	x(-7.5±0.7) ^a	y(-3.3±1.7) ^b				
b*									
0		x(17.9±1.3) ^a	x(17.9±1.3) ^a	x(17.9±1.3) ^a	x(17.9±1.3) ^a	x(17.9±1.3) ^a	x(17.9±1.3) ^a	x(17.9±1.3) ^a	x(17.9±1.3) ^a
1		x(20.6±1.8) ^a	x _y (20.4±2.5) ^a	y(23.4±1.4) ^a	y(22.7±3.4) ^a	y(20.8±0.9) ^a	x _y (21.7±1.8) ^a	x _y (22.3±2.6) ^a	y(24.2±2.8) ^a
3		x(20.0±1.0) ^a	y(23.7±1.4) ^a	y(22.1±2.9) ^a	y(23.1±1.1) ^a	z(28.1±1.2) ^a	y(25.5±3.1) ^a	y(26.0±4.6) ^a	y(24.8±1.0) ^a
7		y(24.8±1.0) ^a	y(22.4±1.6) ^a	y(24.3±3.2) ^a	y(22.6±2.0) ^a				
<i>Reserve Side</i>		4°C				10°C			
Day/Conc (μL/L)		0	40	100	400	0	40	100	400
L*									
0		x(46.5±1.1) ^a	x(46.5±1.1) ^a	x(46.5±1.1) ^a	x(46.5±1.1) ^a	x(46.5±1.1) ^a	x(46.5±1.1) ^a	x(46.5±1.1) ^a	x(46.5±1.1) ^a
1		x _y (48.6±2.6) ^a	x _y (49.2±2.1) ^a	y _z (50.3±0.5) ^a	y(50.2±2.6) ^a	y(51.3±3.3) ^a	x _y (48.9±3.1) ^a	y(49.7±1.9) ^a	y(51.2±2.0) ^a
3		x _y (49.1±2.3) ^a	y(51.4±2.9) ^a	z(51.7±0.8) ^a	y(51.5±1.7) ^a	y(51.9±2.0) ^a	y(53.2±0.9) ^a	y(51.0±1.6) ^a	y(52.3±1.2) ^a
7		y(52.0±1.2) ^a	y(51.3±0.8) ^a	x _z (48.7±0.5) ^a	y(51.1±1.5) ^a				
a*									
0		x(-9.1±0.3) ^a	y(-9.1±0.3) ^a	x(-9.1±0.3) ^a	x(-9.1±0.3) ^a	x _y (-9.1±0.3) ^a	x(-9.1±0.3) ^a	x(-9.1±0.8) ^a	x(-9.1±0.8) ^a
1		x(-9.1±0.4) ^a	x _y (-8.3±0.8) ^{a,b}	y(-8.3±0.7) ^{a,b}	y(-7.8±0.6) ^b	x(-8.5±0.8) ^a	x(-9.3±0.2) ^a	x(-8.7±0.6) ^a	x(-8.9±0.5) ^a
3		x(-9.4±0.7) ^a	x _y (-8.7±1.0) ^{a,b}	y(-8.1±0.2) ^{a,b}	y(-7.5±0.5) ^b	y(-9.6±0.4) ^a	y(-6.8±0.8) ^b	y(-6.1±-0.7) ^{b,c}	y(-4.5±1.3) ^c
7		x(-8.8±0.2) ^a	x(-7.5±0.8) ^b	y(-7.9±0.3) ^{a,b}	z(-3.1±0.9) ^c				
b*									
0		x(24.5±1.3) ^a	x(24.5±1.3) ^a	x(24.5±1.3) ^a	x(24.5±1.3) ^a	x(24.5±1.3) ^a	x(24.5±1.3) ^a	x(24.5±1.3) ^a	x(24.5±1.3) ^a
1		x(26.3±1.8) ^a	x(25.6±2.1) ^a	y(27.0±0.8) ^a	x(23.9±2.1) ^a	x(24.4±1.3) ^a	x(26.8±1.6) ^a	x(25.5±1.2) ^a	x(25.0±1.3) ^a
3		x(25.6±2.5) ^a	x(27.1±0.6) ^a	x _y (26.3±1.0) ^a	x(23.6±1.1) ^a	x(26.4±1.1) ^a	x(24.5±1.5) ^a	x(22.4±2.6) ^a	x(25.0±2.2) ^a
7		x(26.4±1.2) ^a	x(25.0±2.0) ^a	x _y (26.6±0.5) ^a	x(23.3±1.6) ^a				

^{a-c} Mean color values within a row (treatment concentration) sharing a common superscript letter in the same row are not significantly different ($P>0.05$)

^{x-z} Mean color values within a column (storage day) sharing a common subscript letter in the same column are not significantly different ($P>0.05$)

Table 3.3. Sensory scores of control and EP-treated green onions stored at 4°C and 10°C.

Day/Conc(μL/L)	4°C				10°C			
	Color				Odor			
	0	40	100	400	0	40	100	400
0	x(7.5±1.4) ^a	x(7.5±1.4) ^a	x(7.5±1.4) ^a	x(7.5±1.4) ^a	x(7.5±1.4) ^a	x(7.5±1.4) ^a	x(7.5±1.4) ^a	x(7.5±1.4) ^a
1	x _y (6.6±1.2) ^{a,b}	y(6.0±1.8) ^{a,b}	x _y (7.0±1.1) ^a	y(5.7±1.7) ^b	x(7.1±1.3) ^a	x _y (7.0±1.3) ^a	y(5.6±1.2) ^b	y(5.7±1.7) ^b
3	y(6.3±1.3) ^a	x _y (6.5±1.3) ^a	y _z (6.5±1.1) ^a	y(5.8±1.2) ^a	x(7.0±1.2) ^a	y(6.0±1.2) ^{a,b}	y(4.8±1.7) ^c	y(5.5±1.8) ^{b,c}
7	y(6.3±1.2) ^a	y(6.3±1.0) ^a	z(5.7±1.3) ^a	y(5.9±1.4) ^a				
	Odor				Texture			
0	x(6.6±1.6) ^a	x(6.6±1.6) ^a	x(6.6±1.6) ^a	x(6.6±1.6) ^a	x(6.6±1.6) ^a	x(6.6±1.6) ^a	x(6.6±1.6) ^a	x(6.6±1.6) ^a
1	x _y (5.9±1.2) ^a	x _y (5.9±2.0) ^a	x _y (5.9±1.2) ^a	x(5.7±1.2) ^a	x(5.9±1.2) ^a	x _y (5.9±1.4) ^a	x _y (5.7±1.4) ^a	x _y (5.6±1.5) ^a
3	y(5.5±1.4) ^a	y(5.3±1.2) ^a	x _y (6.1±1.3) ^a	x(5.4±1.7) ^a	x(5.7±1.2) ^a	y(5.4±1.2) ^a	y(5.2±1.9) ^a	y(5.1±1.9) ^a
7	x _y (5.6±1.7) ^a	x _y (5.8±1.2) ^a	y(5.3±1.7) ^a	x(5.4±1.8) ^a				
	Overall Quality							
0	x(7.4±1.2) ^a	x(7.4±1.2) ^a	x(7.4±1.2) ^a	x(7.4±1.2) ^a	x(7.4±1.2) ^a	x(7.4±1.2) ^a	x(7.4±1.2) ^a	x(7.4±1.2) ^a
1	y(6.3±1.1) ^a	y(6.0±1.6) ^a	x _y (6.6±0.8) ^a	y(5.8±1.4) ^a	x _y (6.7±1.0) ^a	x _y (6.5±1.1) ^a	y(5.8±1.4) ^a	y(5.6±1.5) ^a
3	y(6.0±1.1) ^a	y(6.0±0.9) ^a	y(6.3±1.1) ^a	y(5.5±1.2) ^a	y(6.3±1.0) ^a	y(5.8±1.2) ^{a,b}	y(4.9±1.8) ^b	y(5.2±1.7) ^{a,b}
7	y(6.1±1.6) ^a	y(5.7±1.0) ^{a,b}	z(5.4±1.4) ^{a,b}	y(4.8±1.2) ^b				

Sensory scores were assigned by panelists using a 9-point hedonic scale: 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely.

^{a-c} Mean sensory scores within a row (treatment concentration) sharing a common superscript letter in the same row are not significantly different ($P>0.05$)

^{x-z} Mean sensory scores within a column (storage day) sharing a common subscript letter in the same column are not significantly different ($P>0.05$)

While headspace EP concentrations increased with storage time at 10°C, EP concentrations were maintained over a longer period in containers stored at 4°C.

The concentration of EP in the headspace of containers containing green onions and spinach declined significantly over 7 days of storage at 4°C and over 5 days of storage at 10°C to less than <5 μg/L in the green onion and baby spinach containers (Figure 3.4 and Figure 3.5).

Table 3.4. Sensory scores of control and EP-treated baby spinach leaves stored at 4°C and 10°C.

Day/Conc (μL/L)	4°C				10°C			
	0	40	100	400	0	40	100	400
Color								
0	x(7.5±0.8) ^a	x(7.5±0.8) ^a	x(7.5±0.8) ^a	x(7.5±0.8) ^a	x(7.5±0.8) ^a	x(7.5±0.8) ^a	x(7.5±0.8) ^a	x(7.5±0.8) ^a
1	x(7.1±1.6) ^a	x,y(6.5±1.6) ^{a,b}	y(5.7±1.8) ^b	y(5.4±1.7) ^b	x(6.9±1.4) ^a	y(5.7±1.8) ^b	y(4.1±1.6) ^c	y(3.7±1.6) ^c
3	y(5.4±1.7) ^a	y,z(5.5±1.6) ^a	z(4.6±1.2) ^{a,b}	y,z(4.3±1.7) ^b	y(5.7±1.6) ^a	z(3.4±1.7) ^b	y(3.9±1.6) ^b	z(2.7±1.6) ^b
7	x,y(6.5±2.4) ^a	z(4.5±2.3) ^b	y,z(4.8±2.4) ^{a,b}	z(3.4±1.7) ^b				
Odor								
0	x(6.0±1.2) ^a	x(6.0±1.2) ^a	x(6.0±1.2) ^a	x(6.0±1.2) ^a	x(6.0±1.2) ^a	x(6.0±1.2) ^a	x(6.0±1.2) ^a	x(6.0±1.2) ^a
1	x(5.7±1.0) ^a	x(5.6±1.1) ^a	x,y(5.4±1.5) ^a	x,y(5.0±1.2) ^a	x(5.7±1.2) ^a	x(5.5±1.2) ^{a,b}	y(4.6±1.6) ^b	y(4.5±1.4) ^b
3	x(5.3±1.2) ^a	x,y(5.3±1.2) ^a	y,z(4.8±1.3) ^a	y,z(4.4±1.7) ^a	x(5.4±1.2) ^a	y(4.1±2.0) ^{a,b}	y(4.3±1.4) ^{a,b}	y(3.6±2.0) ^b
7	x(5.1±1.7) ^a	y(4.4±1.1) ^{a,b}	z(4.3±1.1) ^{a,b}	z(3.4±1.9) ^b				
Texture								
0	x(6.4±1.7) ^a	x(6.4±1.7) ^a	x(6.4±1.7) ^a	x(6.4±1.7) ^a	x(6.4±1.7) ^a	x(6.4±1.7) ^a	x(6.4±1.7) ^a	x(6.4±1.7) ^a
1	x(6.3±1.9) ^a	x,y(5.4±1.9) ^{a,b}	y(4.3±1.7) ^{b,c}	y(3.9±1.4) ^c	x(6.4±1.3) ^a	y(4.85±1.6) ^b	y(4.0±1.5) ^{b,c}	y(3.4±1.6) ^c
3	y(4.1±1.4) ^a	y,z(4.2±1.4) ^a	y(3.9±1.5) ^a	y(3.8±1.6) ^a	y(4.7±1.6) ^a	z(3.3±1.6) ^b	y(3.1±1.4) ^b	y(2.9±1.5) ^b
7	x,y(4.9±2.4) ^a	z(3.2±1.7) ^b	y(3.3±2.1) ^{a,b}	z(2.5±1.3) ^b				
Overall Quality								
0	x(7.1±0.9) ^a	x(7.1±0.9) ^a	x(7.1±0.9) ^a	x(7.1±0.9) ^a	x(7.1±0.9) ^a	x(7.1±0.9) ^a	x(7.1±0.9) ^a	x(7.1±0.9) ^a
1	x,y(6.5±1.5) ^a	x(6.1±1.3) ^{a,b}	y(4.9±1.6) ^{b,c}	y(4.6±1.6) ^c	x(6.5±1.2) ^a	y(5.4±1.6) ^b	y(4.05±1.4) ^c	y(3.8±1.4) ^c
3	z(4.8±1.5) ^a	y(4.8±1.5) ^a	y(3.9±1.3) ^a	y,z(4.1±1.6) ^a	y(5.1±1.6) ^a	z(3.3±1.5) ^b	y(3.6±1.4) ^b	y(2.9±1.6) ^b
7	y,z(5.4±2.2) ^a	y(3.8±1.6) ^b	y(3.9±1.9) ^{a,b}	z(2.9±1.4) ^b				

Sensory scores were assigned by panelists using a 9-point hedonic scale: 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely.

^{a-c} Mean sensory scores within a row (treatment concentration) sharing a common superscript letter in the same row are not significantly different ($P>0.05$)

^{x-z} Mean sensory scores within a column (storage day) sharing a common subscript letter in the same column are not significantly different ($P>0.05$)

DISCUSSION

This study explored the use of gaseous EP as an alternative produce safety intervention method that could be used for the inactivation of *E. coli* O157:H7 on green onions and baby spinach. Samples were dip-inoculated to determine the efficacy of gaseous EP on surface pathogens as well as pathogens that may have infiltrated the plant tissue through cut and damaged areas. Gaseous EP treatments at concentrations of 100 and 400 μL/L were effective at reducing *E. coli* O157:H7_(GFP) populations significantly and demonstrated excellent antimicrobial properties. The antimicrobial properties of gases generally depend on extrinsic factors such as the gas concentration, headspace volume, exposure time, relative

humidity, and treatment temperature. In this study, EP inhibition of *E. coli* O157:H7_(GFP) was more effective with increasing EP concentration, storage temperature, and time. The lowest concentration of gaseous EP (40 µL/L) did not significantly reduce the population of *E. coli* O157:H7_(GFP) on green onions or baby spinach relative to the control samples.

In contrast, higher concentrations of gaseous EP (100 and 400 µL/L) demonstrated a faster and more effective inactivation of *E. coli* O157:H7_(GFP) on green onion or spinach samples stored at 10°C. An approximate 5.0 log CFU/g reduction of *E. coli* O157:H7_(GFP) on green onions stored at 4°C was achieved in 7 days; only 5 days were required to obtain a similar log reduction when samples were stored at 10°C. Similarly, a 4.3 log CFU/g reduction of *E. coli* O157:H7_(GFP) was observed in baby spinach samples stored at 4°C storage for 7 days, and a higher inactivation was achieved after 5 days of storage at 10°C (Table 3.1). Enhanced inactivation at higher temperatures has also been reported for gaseous allyl isothiocyanate, carvacrol, and cinnamaldehyde applications, which have been reported to resulted in >4 log reduction of *E. coli* O157:H7 on lettuce and spinach leaves within 4 days of storage at 0°C and 4°C and within 2 days on samples stored at 10°C (28). Allyl isothiocyanate has also been reported to reduce *Salmonella* populations on sliced tomatoes by 1.0 and 3.5 log CFU/g within 10 days at storage temperatures of 4°C and 10°C, respectively (29).

It has been reported that *E. coli* O157:H7 can survive for an extended period of time on leaf lettuce exposed to pathogens through spray, surface irrigation (34, 35) or manure composts (16). Recent studies have indicated a significant increase in the pre-harvest populations of *E. coli* O157:H7 in the phyllosphere of young lettuce plants (6) and packaged or damaged leaves of spinach, lettuce, cilantro, and parsley if they are stored at ≥8°C (18, 22). However, in other studies, *E. coli*

O157:H7 populations did not change significantly on lettuce or spinach stored at 4°C to 5°C (30). Therefore, any contaminated fresh produce is at risk for contamination because of potential temperature abuse in the post-harvest distribution chain. Because EP was more effective at 10°C than at 4°C in this study, it could control *E. coli* O157:H7 growth in the event of temperature abuse. In the absence of EP (control samples), *E. coli* O157:H7_(GFP) populations also decreased in both green onions and baby spinach stored at 4°C and 10°C. However, the decrease was greater at 4°C than at 10°C. Because the storage containers used in this study were not airtight, it is possible that small amounts of EP may have leaked into control containers. Although control and EP-treated containers were separated from each other, all containers were stored in the same cold room (400 × 150 ft).

The maximum headspace concentrations of EP occurred after 12 h of storage with 400 µL/L EP at 4°C and 10°C, and the concentrations declined thereafter, suggesting that the EP was rapidly volatilized in the closed containers. Temperature had a dramatic effect on the volatilization of EP into the headspace, with higher headspace EP concentrations in containers stored at 10°C than at 4°C. However, EP concentrations remained more stable over time during storage at 4°C. Nadarajah and Holley (2004) also reported better preservation of gaseous allyl isothiocyanate in the headspace of packaged ground beef stored at 4°C than at 10°C (24). In this study, EP concentrations declined gradually over storage time, and greater inactivation of *E. coli* O157:H7 was observed at the higher storage temperature. Therefore, it is possible that EP may have been absorbed by green onion and spinach tissue and continued interacting with the bacteria during the entire storage period.

Gaseous EP treatments demonstrated excellent antimicrobial properties against *E. coli* O157:H7, and EP is already recognized as GRAS for the direct addition to food for human consumption. While the EP treatments tested minimally changed the sensory attributes of green onions, the conditions tested were not appropriate for baby spinach. Thus, EP interactions with different produce items need to be investigated, and the optimum treatment and delivery conditions should be adjusted for different types of fresh produce. Our results indicate that gaseous EP treatment was effective in reducing the *E. coli* O157:H7_(GFP) population on dip-inoculated green onions while minimally affecting their sensory attributes.

Dip-inoculation of produce facilitated the infiltration of *E. coli* O157:H7_(GFP) into green onions and baby spinach through the stomata, cuts, damaged leaves, and other open areas in addition to the surface contamination. Since the highest concentration of EP treatment (400 µL/L) resulted in more than a 4.7 log reduction in the *E. coli* O157:H7_(GFP) population and reduced the pathogen population below the limit of detection (<0.5 log) on green onions on day 7 at 4°C and on day 5 at 10°C, it could be assumed that gaseous EP was also effective on pathogens that had infiltrated the plant tissue. Once pathogens are attached, infiltrated, and/or localized in protected areas such as stomata and within the tissue, washing with deionized water and chlorine may have no or very little effect on their removal (38). Beuchat *et al.* (1999) demonstrated that sprays of 200 ppm chlorine or deionized water alone were equally effective on reducing *E. coli* O157:H7 populations on lettuce (3). Similar results have also been reported for lactic acid, phytic acid, calcinated seashells, and chitosan, which showed no significantly higher pathogen reduction than produce washed with water alone (15). These results suggest that gaseous EP is more effective than most of the chlorine or non-chlorine based surface disinfectants, where the inactivation efficacies are less than 2 log units of foodborne pathogens on most of the fresh produce (2). Thus, EP

could be considered as an alternative gaseous antimicrobial agent where the surface decontamination has limited efficacy.

Other gaseous antimicrobials have been used for the decontamination of the fresh produce. Gaseous chlorine dioxide (ClO_2) has been shown to significantly reduce *Salmonella enterica*, *E. coli* O157:H7, *L. monocytogenes* populations on the skin of surface-inoculated cabbage, carrots, lettuce, apples, tomatoes, onions, and peaches (37). However, prolonged exposure to chlorine vapors can cause skin and respiratory tract irritation (2). The ozone-based SanVac process using optimized parameters resulted a 1.8 log CFU/g *E. coli* O157:H7 reduction on spot-inoculated spinach leaves without affecting the sensory attributes of the spinach (40). Ozone may cause physiological damage to produce due to its strong oxidation capabilities and can result in corrosion on metal surfaces. In addition, ozone-producing technologies are generally complicated and expensive for most practical applications. Gaseous allyl isothiocyanate (AIT) also effectively reduces pathogen populations on lettuce, spinach, and tomatoes (28, 29). The application of 16 $\mu\text{L/L}$ AIT resulted in 4.42 and 4.39 log CFU/leaf reductions of *E. coli* O157:H7 on lettuce and spinach leaves, respectively, after storage at 0°C for 4 days (28). Even though it was stated that this concentration of AIT did not adversely affect the sensory properties of lettuce and spinach, a comprehensive sensory analysis was not performed. However, AIT is a strong skin and mucous membrane irritant (12) and has a reported toxicity (LD_{50}) of 339 mg/kg in rats (17).

Overall, this study supports the use of gaseous EP treatment as an effective and novel decontamination method for the inactivation of *E. coli* O157:H7 on green onions. Gaseous EP would be an alternative antimicrobial agent capable of inactivating *E. coli* O157:H7 on fresh produce in areas that surface decontamination methods may not be able to fully access; therefore, this novel

method could help to prevent fresh produce associated outbreaks. This is the first published study of the antimicrobial properties of EP against foodborne pathogens. Additional research needs to be conducted to more fully understand the impact of EP on the sensory attributes of fresh produce and other types of food, devise effective methods for incorporation of gaseous EP in packaging materials, and understand the possible mechanisms of bacterial inhibition by EP.

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CHAPTER 4

Alicyclobacillus spp.

INTRODUCTION

Alicyclobacillus spp. are important spore-forming bacteria that are commonly associated with the spoilage of various juice and beverage products resulting in financial losses to these industries. *Alicyclobacillus* spp. were first isolated from Japanese hot springs in 1967 and classified within the genus *Bacillus* (43). In 1992, based upon 16S rRNA data analysis, as well as their thermoacidophilic growth characteristics and the unique presence of ω -alicyclic fatty acids as their major membrane lipid, the genus *Alicyclobacillus* was proposed (48). *Alicyclobacillus* spp. are also referred to as “Thermo Acidophilic Bacilli” (or Bacteria) (TAB) due their ability to grow and survive under acidic conditions at high temperatures. The genus originally consisted of *Alicyclobacillus acidocaldarius*, *Alicyclobacillus acidoterrestris* and *Alicyclobacillus cycloheptanicus*. Currently, the genus consists of 21 species, two subspecies and two genomic species (17) while only *A. acidoterrestris* (39), *A. acidiphilus* (26), *A. pomorum* (13) have been isolated from juice and beverages associated with spoilage incidents. On the other hand, *A. acidoterrestris*, *A. acidocaldarius*, and *A. acidocaldarius* subsp. *acidocaldarius* are the most frequently isolated species from fruit juices and juice products (11).

The pH of most fruit juices and beverages had previously been thought to be too low (pH < 4.6) to support the growth of spore-forming bacteria (7), thus making existing thermal processing regimes adequate for the safety and shelf stability of juices for an extended period of time at room temperature. Most bacterial spores, including

Clostridium botulinum, cannot germinate under low pH conditions (pH < 4.6) (18). However, with the 1982 discovery of apple juice spoiled by a thermoacidophilic, spore-forming bacteria (8), the juice and beverage industries started to become aware of the potential dangers of *Alicyclobacillus* species. Member of genus *Alicyclobacillus* spp. are able to grow at low pH (2-6.5) and at temperatures of 20-70°C (52).

Splittstoesser *et al.*, (1994) isolated VF and WAC strains from apple juice and apple-cranberry juice blend from the samples which were the source of off-odor complaints (39) and later identified as *A. acidoterrestris* (34). A 1998 survey by the National Food Processors Association demonstrated that 35% of juice manufacturing companies experienced *Alicyclobacillus* spp. related product spoilage (46).

Splittstoesser *et al.*, (1998) found 3 of 33 commercial juice products to contain detectable levels of *Alicyclobacillus* spp. (40). Pettipher *et al.*, (1997) found that 4 of 4 U.S. apple juice samples, 1 of 4 U.S. apple concentrate samples, 0 of 56 U.K. commercial UHT fruit juice products, and 5 of 18 U.K. fresh apple juice products had *Alicyclobacillus* contamination (33). Eiroa *et al.*, (1999) reported that 14.7% of 75 orange juice samples from 11 Brazilian producers contained *Alicyclobacillus* spp. (12) Many acidic juices and concentrates, including apple, orange, cranberry, white grape, cherry, grapefruit, tomato, pear, mango, and pineapple, carbonated fruits drinks, and tomatoes have been found to be contaminated by *Alicyclobacillus* spp. (8, 10-12, 15, 33, 41, 42). *A. acidoterrestris* spores have been isolated from numerous sources that include the surface of raw fruits, orchards, soils, as well as harvesting and processing environments (11, 16, 49). Studies have indicated that not only juice products, but also processing water, sugar, HFCS, and other ingredients used in juice and beverage production could be potential sources for *Alicyclobacillus* spp. contamination in juice and beverages (11, 27).

Fruit juice pasteurization parameters vary from processor to processor but usually take into consideration the physical properties of fruit juice, pH, storage conditions, nature of the container, factory and processing plants, experience with the product, etc (37, 51). For shelf stable juice and beverages, processing temperatures in the range of 85°C to 95°C are targeted for inactivating enzymes, pathogenic foodborne microorganisms and vegetative, non-heat resistant spoilage microorganisms (36, 41). While these thermal processes are insufficient to inactivate thermotolerant spoilage spore formers, they may function as heat activation of *Alicyclobacillus* spores for germination and growth in their favorable acidic environments. Thermal processes required to inactivate heat-resistant sporeformers are potentially damaging to the sensory and nutritional qualities of the product and therefore, not feasible (30, 46). The resistance to conventional pasteurization and hot-fill processing, combined with *Alicyclobacillus* spp. ability to grow at low pH (2-4) conditions, make it a major microbial spoilage concern for the juice and beverage industries (44).

***Alicyclobacillus* spp. related spoilage.** Although *Alicyclobacillus* spp. are not a cause of foodborne illnesses, even a small number of its contamination may result in large volumes of product spoilage (47). Spoilage by *Alicyclobacillus* can be difficult to detect, as it typically only involves off-flavor development and lacks the typical gas production, turbidity, and sediment associated with other microbial spoilage (46). The most common characteristic of *Alicyclobacillus* contamination is a smoky, medicinal, antiseptic off-odor associated with guaiacol (2-methoxyphenol) (20, 29, 33, 42). While guaiacol has a sensory detection threshold at as little as 2 ppb, it was reported that 10⁵ cells/ml of *A. acidoterrestris* was required to produce sufficient guaiacol to produce detectable taint in apple and orange juices (33). Moreover, other halophenols including 2,6-dibromophenol and 2,6-dichlorophenol are off-odor compounds associated with

Alicyclobacillus contamination and spoilage is often described as medicinal, antiseptic or disinfectant-like odor (20). The detection thresholds for 2,6-dibromophenol and 2,6-dichlorophenol are 0.5 ng/L and 6.2 ng/L (52). Since, guaiacol was detected in juices at much higher concentration than the halophenols and it is more volatile, it is considered the major off-odor compound (19). The minimum number of cells capable of producing these compounds at or above the human sensory threshold level, it is well below the visual detection limit for bacterial growth in juice or beverages (33).

The metabolic production pathway of guaiacol in juice and beverages by *Alicyclobacillus* spp. has not been fully elucidated. The most common hypothesis is the production of guaiacol from vanillic acid during ferulic acid metabolism (38). While vanillic acid could be present in the juice due to a possible contamination or as a flavoring ingredient, it could be also naturally derived from plant polymer lignin. Another precursor of guaiacol formation in juices is tyrosine. Tyrosine is found in apple juice at approximately 4.1 µg/ml and 3.4-13.5 µg/ml in orange juice (10). *A. acidoterrestris* is known as the major producer of guaiacol in juice products. *A. acidoterrestris* is the major microbial spoilage concern for the juice and beverage industries as it is wide spread in the environment, and the production of high off-odor compounds that includes guaiacol (44). However, some strains of *A. acidiphilus*, *A. herbarius*, and *A. hesperidum* subs. *aigle* also produce guaiacol and have been found to be associated with off odor formation and product spoilage (26), but their association with juice spoilage is rare (14). On the other hand, researchers have shown that all *A. acidoterrestris* strains were capable of producing guaiacol (14, 52).

Guaiacol production by *A. acidoterrestris* is dependent on the concentration of germinated spores in juice, strain type, oxygen concentration, storage conditions,

thermal pasteurization application practices, and presence and concentration of guaiacol precursors (3, 14). Goto *et al.*, (2008) showed that guaiacol production was varied among *Alicyclobacillus* strains and categorized them into three groups based on their guaiacol production rates in YSG broth supplemented with vanillin. The guaiacol concentrations groups were defined as group I < 10 ppm within 10 h, group II < 10 ppm within 5 h, and group III > 10 ppm within 5 h. However, all the strains demonstrated around 40-50 ppm after the incubation period of 48 h (14). Yokota *et al.*, (2007) also showed there is great diversity of guaiacol production among *A. acidoterrestris* strains in YSG broth supplemented with vanillin. While *A. acidoterrestris* strains B2065 and DSM 2498 were capable of producing 40 and 50 ppb guaiacol, respectively, strains DSM 3923 and DSM 3924 produced 370 and 500 ppb after 72 h incubation (52). Orr *et al.*, (2000) demonstrated that the growth of *A. acidoterrestris* strains (N-1096, N-1100, N-1108, OS-CAJ, SAC) in apple juice was very slow as the number of spores and vegetative cells of *A. acidoterrestris* remained constant during 61-day of storage period at either 21°C or 37°C. However, a sensory panel detected guaiacol in five samples stored at 21°C to 37°C for 8 to 61 days in which the guaiacol was not able to be detected by chromatographic analyses (29).

Thermal resistance. Heat resistance of microorganisms is measured and defined as the decimal reduction time (D-value). This value is the time in minutes required to kill 90% of cells at a given temperature. The D-value can be determined through an experimental survival curve which the log of D is plotted against the heating temperatures. The slope of this curve is the z-value which is defined as the number of degrees (Celsius or Fahrenheit) that temperature has to be increased to cause a 1 log (90%) reduction in D-values (25). Therefore, the inactivation rate of specific microorganisms is best described by its D and z-values. The genus *Alicyclobacillus*

spp. demonstrates a great diversity in their heat resistance. Eiroa *et al.*, (1999) has reported that *A. acidoterrestris* strains DSM 2498, 145, 046, and 070 showed varying heat resistance in orange juice (pH 3.15, 9.0°Brix). The D values ranged from 60.8 to 94.5 min at 85°C, 10.0 to 20.6 min at 90°C, and 2.5 to 8.7 min at 95°C (12). The others reported D_{95°C}-values of *A. acidoterrestris* strains in various juices ranged from 0.1 to 27.8 min. and z-values ranged from 7.2 to 16.4 min. (9, 12, 27, 32, 36, 39). Pontius *et al.*, (1998) showed that VF and WAC strains of *A. acidoterrestris* had similar heat resistance under acidic conditions, while IP strain had a lower heat resistance in the same environment. In addition, the type of organic acid (malic, citric, tartaric) did not significantly affect the heat resistance (34). The thermal heat resistance of *A. acidoterrestris* was mostly affected by temperature (85-97°C), followed by the soluble solid (5-60°Brix), and lastly by pH (2.5-6.0) of the juice and beverages (24, 35). Bahceci and Acar (2007) found the effect of temperature on D-values was three times higher than those of pH (4).

Silva *et al.*, (1999) showed that increasing soluble solid content (°Brix) of blackcurrant concentrate from 26.1 to 58.5 °Brix, increased *A. acidoterrestris* (NCIMB 13137) spores D_{91°C}-values from 3.8 to 24.1 minutes (35). Similarly, an increase in Concord grape juice °Brix from 16 to 65 resulted in an increase of *A. acidoterrestris* strain WAC D_{90°C} value from 11 to 127 min. and D_{95°C} from 1.9 to 12 min (40). However, there was no significant difference in thermal resistance of *A. acidoterrestris* when between 10 and 20 °Brix in apple juice, orange juice, and malt extract broth (9). Splittstoesser *et al.*, (1994) demonstrated that *A. acidoterrestris* VF strain was not able to grow at Riesling grape juice of 21.6°Brix, the dilution of the grape juice to 16°Brix resulted in maximal growth (39). The temperature was also reported to be the greatest effect on D-values as a 2°C temperature increase in

temperature resulted in a considerable decrease on D-value from 2.82 to 0.57 min in cupuaçu extract (35). Researchers suggested that the possession of unique ω -alicyclic fatty acids in the membrane of *Alicyclobacillus* spp. stabilize the membrane and influence the permeability; therefore associated with the exceptional heat resistance of *Alicyclobacillus* spp. and their ability to survive in acidic conditions (21). A study with ω -alicyclic fatty acid deficient *A. acidocaldarius* strains demonstrated that these mutants were more sensitive to heat shock and ethanol than the wild type (23).

Isolation and enumeration. *Alicyclobacillus* spp. cannot grow in rich media that supports the growth of many aerobic bacteria. Splittstoesser *et al.*, (1994) demonstrated nutrient agar (NA), trypticase soy agar (TSA), brain heart infusion (BHI) agar or veal infusion agar did not support the growth of *A. acidoterrestris* spores even if they were acidified to pH 3.5 with tartaric acid (39). However, several media have been successfully used for the isolation and enumeration of *Alicyclobacillus* spp. These media include but are not limited to potato dextrose agar (PDA), orange serum agar (OSA), K-agar, yeast-starch-glucose (YSG)-agar, and *Alicyclobacillus* medium (ALI), Hiraishi glucose yeast extract (HGYE) agar, *Bacillus acidocaldarius* medium (BAM), *Bacillus acidoterrestris* thermophilic (BAT), and SK Agar. Witthuhn (2007) *et al.*, has compared these five isolation media for the recovery of *Alicyclobacillus* spp. from diluted and undiluted juice concentrates and found that PDA (pH 3.7) and OSA (pH 5.5) at 50°C yielded the highest recovery of vegetative cells and endospores of *Alicyclobacillus* spp (50). Pettipher *et al.*, (1997) also compared BAM, PDA, and OSA media and showed that OSA gave the highest recovery while spread plating was found to be more effective than pour plating at 44°C after 48 h (33). It was also shown that *A. acidoterrestris* were able to grow over

a range of temperatures between 25°C to 44°C (33). Murray *et al.*, (2007) evaluated 10 agar media for their suitability to support *Alicyclobacillus* spore germination by testing six strains of *A. acidoterrestris*, three strains of *A. acidocaldarius*, and one strain of *A. cycloheptanicus* (28). K agar, ALI agar, and BAT agar recovered the highest numbers of spores at 50°C incubated for 3-5 days, while OSA and HGYE agar were the least suitable (28).

Intervention methods for *Alicyclobacillus* spp. These studies have provided evidence to support the belief that *Alicyclobacillus* spp. contamination is a widespread problem. *Alicyclobacillus* spp. presence is ubiquitous in nature, their contamination with the juice and beverages are unavoidable. Since current juice pasteurization practices are not sufficient to inactivate *Alicyclobacillus* spp. spores, it is important to understand the prevalence of *Alicyclobacillus* spp. in juices and juice production environments as well as characterizing the thermal resistance and off-odor production capabilities of targeted *A. acidoterrestris* strains. Silva *et al.*, (2001, 2004) and Goto *et al.*, (2008) suggested using *A. acidoterrestris* spores as the target microorganism for the juice pasteurization process in acidic beverages (14, 36, 37). However, the thermal processes required to inactivate all *A. acidoterrestris* spores may reduce nutrient content of juice and beverages dramatically, and change the fresh-like sensory qualities of the product and therefore, is not feasible (30, 46). Moreover, the application of uncontrolled increases in pasteurization temperature may enhance the stimulation *Alicyclobacillus* spp. spores germination and lead to higher incidence of product spoilage. In addition, consumer's expectations and desire for minimally thermal treated beverages have motivated juice manufacturers to better understand the safety and quality risk factors and optimize a balanced heat treatment for the juice and beverages processing. Thus, strict quality parameters, fruit selection, and proper

washing, and optimizing pasteurization conditions should be incorporated into juice processing regimes, in order to reduce *Alicyclobacillus* spp. contamination and their associated spoilage in shelf stable juice products. In order to control *Alicyclobacillus* spp. related spoilage in juice and beverages, several intervention methods have been proposed. These methods include addition of nisin and other bacteriocins (22, 32), benzoic acid and benzoate (45), high pressure (1), ultrasound (53), DMDC (2), lysozyme (6), and essential oils (5). Alternatively, *Alicyclobacillus* spp. contaminated juices could be stored at lower temperature as the vegetative cells of *Alicyclobacillus* spp. do not or very slowly grow cool storage or refrigeration temperatures (31).

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CHAPTER 5

IDENTIFICATION AND HAPLOTYPE DISTRIBUTION OF *Alicyclobacillus* spp. FROM DIFFERENT JUICES AND BEVERAGES¹

ABSTRACT

Alicyclobacillus spp. is an important thermoacidophilic, spore-forming spoilage bacterium that is a major concern for beverage and juice industries. In order to develop effective control strategies and adequately address the prevalence of contamination sources, it is necessary to characterize *Alicyclobacillus*' ecology in fruit, juice and beverage production and processing environments.

Alicyclobacillus spp. isolates were collected from juice, beverage, ingredients, and environmental samples over a period of ten years. A total of 141 isolates were characterized as *Alicyclobacillus* spp. by 16S rRNA analysis and the most frequently isolated species was found to be *Alicyclobacillus acidoterrestris* (45 %), *A. acidocaldarius* subsp. *acidocaldarius* (30 %), and *A. acidocaldarius* (11 %).

The majority of thermotolerant sporeformers isolated from apple juices and concentrates was found to be *A. acidoterrestris* (24 out of 36 total apple isolates); while *A. acidoterrestris* was most frequently associated with citrus, citrus concentrates, and their associated environments, isolated by University of Florida (UF) (15 out of total 28 UF citrus isolates). However, *A. acidocaldarius* and subsp. *acidocaldarius* were frequently isolated by Cornell University (CU) (29 out of 35 CU citrus isolates), from citrus juices made from concentrate.

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Four major haplotypes of *Alicyclobacillus* spp. were identified based on the 16S rRNA gene sequencing from the 141 isolates tested. The Allelic Types (ATs) matched the phylogenetic analysis grouping of the different *Alicyclobacillus* spp. based on the isolation source.

Our results suggest a predisposition for certain AT's of *Alicyclobacillus* spp. depending on juice or ingredient isolation source.

INTRODUCTION

Alicyclobacillus spp. are thermoacidophilic, aerobic, spore-forming bacteria first isolated from Japanese hot springs in 1967 and classified within the genus *Bacillus* (31). In 1992, based upon 16S rRNA analysis data, as well as their thermoacidophilic growth characteristics and the unique presence of ω -alicyclic fatty acids as their major membrane lipid, the genus *Alicyclobacillus* was proposed (35).

The pH of fruit juices and beverages had previously been thought to be too low to support the growth of spore-forming bacteria (2), thus making existing thermal processing regimes adequate for product stabilization. However, with the 1982 discovery of apple juice spoiled by a thermoacidophilic, spore-forming bacteria (3), the juice and beverage industries became aware of the potential dangers of *Alicyclobacillus* species. *Alicyclobacillus* spp. are soil-borne bacteria (5, 11, 15, 16) that have been isolated from hot springs (4), fruit and acidic fruit juices, concentrates, and beverages. Many acidic juices and concentrates, including apple, orange, cranberry, white grape, cherry, grapefruit, tomato, pear, mango, and pineapple have been found to be contaminated by *Alicyclobacillus* (3, 7, 13, 24, 28, 29, 36). For shelf

stable juice and beverages, processing temperatures in the range of 85 to 95°C are targeted for inactivating pathogenic foodborne microorganisms and vegetative, non-heat resistant spoilage microorganisms (27, 28). These thermal processes are insufficient to inactivate thermotolerant spoilage sporeformers. Thermal processes required to inactivate heat-resistant sporeformers are potentially damaging the sensory and nutritional qualities of the product and therefore, not feasible (23, 33). The resistance to conventional pasteurization and hot-fill processing, combined with *Alicyclobacillus* spp. ability to grow at low pH (2-4) conditions, make it a major microbial spoilage concern for the juice and beverage industries (32).

Spoilage by *Alicyclobacillus* can be difficult to detect, as it typically only involves off-flavor development and lacks the typical gas production, turbidity, and heavy sediment associated with other microbial spoilage (33). The most common characteristic of *Alicyclobacillus* contamination is a smoky, medicinal, antiseptic off-odor associated with guaiacol (17, 22, 24, 29), which has a sensory detection threshold at as little as 2 ppb (24). In addition, other halophenols including 2,6-dibromophenol and 2,6-dichlorophenol are also associated with *Alicyclobacillus* contamination (17). The minimum number of cells capable of producing these compounds at or above the human sensory threshold level, is well below the visual detection limit for bacterial growth in juice or beverages (24). Therefore, in order to prevent *Alicyclobacillus* contamination and predict the spoilage potential of these contaminants, comprehensive microbiological laboratory analyses are required.

A 1998 survey by the National Food Processors Association demonstrated that 35% of juice manufacturing companies experienced *Alicyclobacillus* spp. related product spoilage (33). Splittstoesser *et al.* (1998) found 3 of 33 commercial juice products to

contain detectable levels of *Alicyclobacillus* spp. (29). Pettipher *et al.* (1997) found that 4 of 4 U.S. apple juice samples, 1 of 4 U.S. apple concentrate samples, 0 of 56 U.K. commercial UHT fruit juice products, and 5 of 18 U.K. fresh apple juice products had *Alicyclobacillus* contamination (24). Eiroa *et al.* (1999) found that 14.7% of 75 orange juice samples from 11 Brazilian producers contained *Alicyclobacillus* (7). Finally, several separate studies have indicated that not only juice products, but also processing water from juice production facilities and soil from the orchard environment can be contaminated with *Alicyclobacillus* (21, 36). These studies have provided evidence to support the belief that *Alicyclobacillus* spp. contamination is a widespread problem within the juice and beverage industry, but prevalence of this contamination has not been thoroughly investigated. In this paper, a typing method based on the 16s rRNA hypervariable region that is capable of differentiating *Alicyclobacillus* spp. from varying sources is presented. The isolates used in this study were collected from juice, beverage, ingredients, and environmental samples at Cornell University (CU) and the University of Florida (UF) over a period of ten years.

MATERIALS AND METHODS

Sample acquisition. A total of 141 *Alicyclobacillus* spp. isolates were characterized from various domestic and international sources. Strains were isolated from apple (36 isolates) and citrus (63 isolates) juices, juice concentrates, processing (wash waters, conveyers) and harvesting environments (citrus grove soil). The remaining 42 isolates were collected from various types of other beverages and concentrates including: sports beverage (6); fruit juice nectar (5); mango puree (2) and concentrate (1); low acid beverages (1); peach tea (1) and concentrate (1); fruit soda (1); mint tea (1); agave syrup (1); pear (1) and blueberry concentrate (1); as well as juice ingredients such as

sugar (18) and High Fructose Corn Syrup (HFCS) (2 isolates). The *Alicyclobacillus* spp. isolates besides apple and citrus juices/concentrates and their associated environments were listed under the “others” category; detailed information about the various isolation sources of the tested *Alicyclobacillus* spp. can be accessed at:

<http://www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S0168160510003764/1-s2.0-S0168160510003764-mmcl.pdf/271249/html/S0168160510003764/497e0a268e61d1e520a471279bb38214/mmcl.pdf>

Isolation of acidothermophilic sporeformers. Acidothermophilic spore-forming *Alicyclobacillus* spp. were isolated by membrane filtration, media plating, and enrichment from the tested samples as described below. One liter of sample product was vacuum filtered through 0.22 μ m nitrocellulose filter membrane (Millipore, Bedford, MA) and the filter aseptically transferred either to acidified Potato Dextrose Agar (PDA) or ALI agar (36), pH 3.5 or 5.6. These plates were incubated aerobically for 5 days at 50°C. Small, flat, light brown, dull surface, round, smooth-edged colonies typical of the *Alicyclobacillus* spp. colony morphology were then enumerated and expressed as CFU/liter. Alternatively, for concentrates, 100 g of juice or sugar concentrate was diluted to a final volume of 1 liter with sterile distilled water and treated as above, with enumeration being expressed as CFU/100 g concentrate. In some cases, due to sample viscosity or debris, the full 1 liter was unable to be filtered using a single filter. In such instances, either multiple membranes were utilized or the filterable volume was recorded and the colony counts extrapolated for expression as CFU/liter or CFU/100 g as appropriate. These samples, unless otherwise indicated, represent typical juice and beverage samples as opposed to suspect contaminated samples. For samples containing pulp or insoluble solids, enrichment for *Alicyclobacillus* spp. was performed with 25 g sample with 225 ml of Potato Dextrose Broth (PDB) or Ali broth and incubated at 50°C for 7 days. A 100 μ l of the sample

enrichment was plated onto PDA or Ali agar for isolation of individual colonies and subsequent identification.

DNA preparation. Selected putative *Alicyclobacillus* from the isolations were aseptically transferred to non-acidified Potato Dextrose Broth (PDB) pH 5.6 and incubated at 50°C for 3-4 days. DNA lysates for PCR amplification were prepared from these cultures as described by Furrer *et al.* (1991) with slight modifications. Briefly, 500 µl of broth was centrifuged at 13,800 g for 10 minutes. The pellets were then frozen at -80°C for 15 min. and later resuspended in 95 µl of 1X PCR buffer (Fisher Scientific, Suwanee, GA). Lysozyme (Sigma, Saint Louis, MO) was added to achieve a final concentration of 2.5 mg/ml. After 15 min. of incubation at room temperature (25°C), 2 µl of proteinase K (20 mg/ml, Sigma, Saint Louis, MO) was added and the mixture incubated at 58°C for 1 h. Enzymes were subsequently inactivated by heating at 95°C for 8 minutes.

16S rRNA gene analysis. The 16S rRNA gene was amplified by PCR using universal forward (5'-AGAGTTTGATCCTGGCTCAG-3') primer (34) and a reverse primer (5'-GGTATCTAATCCTGTTTGC-3') which was the complementary sequence of a previously published primer (25). PCR was carried out under the following conditions: 1 cycle of denaturing at 94°C for 3 min., 30 cycles of denaturing at 94°C for 1 min, annealing at 37°C for 2 min. and extension at 72°C for 1.5 min, and one final cycle of extension at 72°C for 8 min. (Robocycler, Stratagene, La Jolla, CA). The PCR product was purified with Qiagen Gel Extraction (Qiagen Corp., Valencia, CA) kit and sequenced using an ABI Prism 3730 DNA sequencer (Applied Biosystems, Foster City, CA) at Cornell University Life Science Core Laboratories Center (Ithaca, NY). The resulting 16s rRNA gene sequences were used for homology comparison using

the NCBI BLAST nucleotide search database (<http://blast.ncbi.nlm.nih.gov/blast.cgi>).

Haplotype analysis. 16s rRNA gene sequences for all of the strains were aligned in MegAlign[®] (DNASTar[®], Lasergene). DnaSP version 5.0 (26) was used for descriptive analyses, including allele assignment. Different alleles were assigned to gene sequences that differed from each other at one or more nucleotide sites. Isolates with 2 different allelic types were considered to represent 2 different molecular subtypes. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with 2000 bootstrap replicates; *Bacillus subtilis* (CU105) and *B. ginsengihumi* (CU106) isolates obtained from juice products were used as out-groups in the 16S rRNA phylogenetic trees. In addition, 16s rRNA gene sequences of ATCC 43030 *A. acidocaldarius* (GenBank accession number: AY573796), ATCC 27009 *A. acidocaldarius* subsp. *acidocaldarius* (GenBank accession number: AB042056), and ATCC 49025 *A. acidoterrestris* (GenBank accession number: AY573797) were included in the phylogenetic tree as reference strains. All phylogenetic analyses were performed using PAUP version 4.0 beta 10.

Statistical analysis. Associations between *Alicyclobacillus* spp. and the source of the various isolates (citrus, apple, other) were examined either by Chi-square analysis or Fisher's exact test. Statistical analyses were performed by S-plus 6.2 (Insightful, Seattle, WA).

RESULTS

Based on the 16S rRNA analysis of 141 *Alicyclobacillus* spp. from various juices, concentrates, and environmental samples, the most frequently isolated species was found to be *Alicyclobacillus acidoterrestris* (64 isolates), followed by *A.*

acidocaldarius subsp. *acidocaldarius* (43 isolates) and *A. acidocaldarius* (16 isolates). In addition, this study isolated “less frequent” *Alicyclobacillus* spp. that consisted of *A. fastidiosus* (9 isolates), *A. sacchari* (5 isolates), *A. hesperidum* (2 isolates), *A. tengchongensis* (1 isolate), and *A. acidiphilus* (1 isolate); see Table 5.1.

Alicyclobacillus spp. were isolated from apple and orange juices/concentrates as well as other types of juices/concentrates and their associated environments. However, *A. acidoterrestris* (24 out of 36 total apple isolates) was more frequently associated with apple juice and concentrate than other *Alicyclobacillus* spp. (Table 5.1).

Alicyclobacillus acidocaldarius subsp. *acidocaldarius* (20 isolates) and *A. acidocaldarius* (9 isolates) were more frequently associated with a total of 35 Cornell University (CU) isolated citrus samples, whereas, *A. acidoterrestris* (15 isolates) was the most frequently isolated species from the total of 23 University of Florida (UF) citrus isolates. The UF *A. acidoterrestris* citrus isolates were isolated 5 times from citrus concentrates (Brazil [4], Florida [1]), 6 times from Florida citrus, 3 times from citrus grove soil (Brazil [1], Florida [2]), and 1 time from Florida citrus wash water. The remaining 8 UF *A. acidoterrestris* strains were isolated 5 times from Fruit Juice Nectar (Brazil [4], South America [1]), 2 times with mango products (concentrate [1], puree [1]), and 1 time from apple juice. An association between the common species (*A. acidoterrestris*, *A. acidocaldarius*, and *A. acidocaldarius* subsp. *acidocaldarius*) and the isolation sources (citrus, apple, others) were not statistically significant for all 141 isolates ($p=0.07$; Table 5.1). A high level of significance ($p=0.0003$) was found between the isolate source and species among the CU isolates. The marked difference between the CU and UF isolates is due to the high incidence of *A. acidoterrestris* in both UF citrus and CU apple samples.

Alicyclobacillus fastidiosus was isolated 6 times from citrus juice and citrus environments, as well as 3 times from sports beverages. Two *A. hesperidum* isolates were identified from citrus juices. *Alicyclobacillus sacchari* was isolated 4 times from citrus samples [grapefruit (1), orange (1), citrus wash water (2)] and one time from imported mango puree. *Alicyclobacillus acidiphilus* and *A. tengchongensis* were each isolated only once from sugar and sports beverages, respectively. The remaining less frequently isolated *Alicyclobacillus* spp. were primarily associated with imported tropical juice concentrates. Fisher's exact test on 5×2 table (five "less frequently isolated species" \times two sources ("citrus" and "others")) showed no significant association ($p=0.3293$) between the less frequently isolated *Alicyclobacillus* spp. and the isolation source (Table 5.1).

An alignment of the 552 bp 16S rRNA gene sequence (representing nt 101 to 652 of the 1514 16S rRNA gene) for all *Alicyclobacillus* species was conducted. Four major allelic types (ATs) were differentiated among the 141 characterized *Alicyclobacillus* spp. isolates that consist of the most frequently isolated species (Figure 5.1). The phylogenetic analysis demonstrated that the *Alicyclobacillus* spp. were clustered into multiple branches while *B. subtilis* and *B. ginsengihumi* juice isolates were clearly separated from the *Alicyclobacillus* isolates. Although the isolated strains were identified under the same species name by 16S rRNA BLAST search, the several species of *Alicyclobacillus* were clustered into different branches on the 16S phylogenetic tree. Particularly, *A. acidocaldarius* and *A. acidocaldarius* subsp. *acidocaldarius* clustered into two different branches under the AT 2 (Table 5.1). While 22 of *A. acidocaldarius* subsp. *acidocaldarius* clustered under the AT 1, the remaining 15 *A. acidocaldarius* subsp. *acidocaldarius* and 12 *A. acidocaldarius* clustered under the AT 2 (Table 5.2). Similarly, *A. acidoterrestris* clustered into 2

major allelic types (AT). AT 3 was represented by 26 isolates, AT 4 represented by 27 isolates. The further phylogenetic characterization of these four major allelic types demonstrated that *A. acidocaldarius* subsp. *acidocaldarius* AT 1 was isolated 16 times from citrus and its processing environments and 6 times from apple juice and concentrates. Interestingly, AT 1 was not isolated from juice and beverage ingredients (sugar, HFCS) or other types of juices. Alternatively, *A. acidocaldarius* subsp. *acidocaldarius* and *A. acidocaldarius* species represented by AT 2, was isolated 12 times from citrus and 12 times from other juices (3 isolates) and juice ingredients (sugar: 7 isolates; HFCS: 2 isolates), but only 3 times from apple juices and concentrates.

Table 5.1. Distribution of *Alicyclobacillus* spp. in apple, citrus, and other juices or concentrates.

Species	Source		
	Apple	Citrus	Others
<i>A. acidocaldarius</i>	4	9	3
<i>A. acidocaldarius</i> subsp. <i>acidocaldarius</i>	8	23	12
<i>A. acidoterrestis</i>	24	19	21
<i>A. fastidiosus</i>	-	6	3
<i>A. sacchari</i>	-	4	1
<i>A. tengchongensis</i>	-	-	1
<i>A. hesperidum</i>	-	2	-
<i>A. acidiphilus</i>	-	-	1
Total	36	63	42

Figure 5.1. 16S rRNA gene-based phylogenetic tree for 141 *Alicyclobacillus* spp. isolates. The tree was constructed using the Neighbor-Joining method with a 552 nucleotide 16S rRNA fragments. *B. subtilis* (CU105) and *B. ginsengihumi* (CU106) were used as out-groups. A total of 2000 bootstrap replications were performed (frequencies <70 % are not shown) (CU: Cornell University; UF: University of Florida).

Fisher's exact test showed a significant association between the AT 1/AT 2 and the source of the isolates ($p=0.0005$). A similar pattern to *A. acidocaldarius* species was observed for *A. acidoterrestris* allelic types. While AT 3 was more frequently associated with citrus (12 isolates), AT 4 was isolated a total of 26 times from apple (15 isolates) juice and other types of juices [mint (1 isolate) and peach (1 isolate) tea; peach (1 isolate) and blueberry (1 isolate) concentrates] as well as juice ingredients (sugar: 7 isolates). Allelic type (AT) 4 was only isolated once from citrus wash water in a citrus processing facility (Table 5.2). The association between the AT 3/AT 4 and the source of the isolates was also found to be significant ($p=0.0009$).

Table 5.2. Distribution of the four major 16S rRNA allelic types (ATs) of *Alicyclobacillus* spp. from different isolation sources.

Source	Allelic Types (ATs)			
	<i>A. acidocaldarius</i> /subsp. <i>acidocaldarius</i>		<i>A. acidoterrestris</i>	
	I	II	III	IV
Apple	6	3	7	15
Citrus	16	12	12	1
Others	0	12	7	11
Total	22	27	26	27

DISCUSSION

Alicyclobacillus acidoterrestris is the predominant acidothermophilic spore former in various juice and beverage products, implying a high incidence of *A. acidoterrestris* in juice production and processing environments. A similarly high frequency of *A. acidoterrestris* isolation from various juice beverages and fruit orchards has also been reported in different studies (11, 32). Thus, *A. acidoterrestris* may be regarded as the most important target species in the genus of *Alicyclobacillus* due to its high frequency of isolation, guaiacol production, and heat tolerance (11, 23).

Alicyclobacillus acidocaldarius subsp. *acidocaldarius* and *A. acidocaldarius* were also frequently isolated from CU citrus samples (Table 5.1), while in UF samples *A. acidoterrestris* is the predominant isolate. While the citrus samples analyzed by UF were generally from Florida citrus harvesting and processing environments as well as from exported citrus concentrates, CU analyzed samples were generally isolated from juices made from concentrate. Not all *Alicyclobacillus* spp. are capable of growing in juice drinks; *A. acidocaldarius*' growth in juice drinks was reported to be none or minimal as compared to other species, including *A. acidoterrestris*, *A. hesperidum*, and *A. acidiphilus* (10, 30). The frequent isolation of these species from CU citrus juice samples might be indicative of *A. acidocaldarius* subsp. *acidocaldarius* and *A. acidocaldarius* contamination of the juice concentrate or during transportation and processing. As presented above, *A. acidocaldarius* subsp. *acidocaldarius* and *A. acidocaldarius* haplotype AT 2 were isolated a total of nine times from beverage ingredients, juices (citrus, apple, and other juices), as well as their harvesting and processing environments. Goto *et al.* (2006) also identified *A. acidocaldarius* in 25 of 27 characterized *Alicyclobacillus* spp. from various juices including orange and lemon juices, orange and apple concentrates, watermelon juice concentrates, soft drinks as

well as raw materials from different regions. The high contamination frequency of *A. acidocaldarius* in various juices from different production regions may also be indicative of contamination during juice processing and handling. These findings indicate that the *Alicyclobacillus* spp. contamination profile may vary depending on the production regions.

The 16S rRNA gene-based subtyping is generally considered to have limited utility due to the relatively low gene diversity within most bacterial species (6, 8). However, Goto and others (2002) demonstrated that the 16S rRNA nucleic acid sequencing includes a hypervariable (HV) region at the 5' end (nt 70 to 344) among *Bacillus* (12) and *Alicyclobacillus* species (9). This HV region was considered as a very efficient index for the identification, distribution, and grouping of *Alicyclobacillus* species. The 552 bp amplicon used in the phylogenetic analysis of this study covers the majority of the HV region and therefore, it is considered to be a sufficient to perform phylogenetic analysis for the isolated *Alicyclobacillus* spp.

The 16S rRNA phylogenetic tree of the isolates in this study indicates the wide distribution of *Alicyclobacillus* spp. in juices and environmental samples. The Allelic Types (ATs) profiling and the statistical analysis suggests that certain ATs of *Alicyclobacillus* spp. are more commonly associated with certain types of juices than others, even though they were listed under the same species name at the taxonomic level. For instance, *A. acidocaldarius* subsp. *acidocaldarius* AT 1 isolates were found to be more commonly associated with citrus products while AT 2 isolates were associated with other types of juices and juice ingredients. In addition, *A. acidoterrestris* AT 3 isolates were associated with citrus products, AT 4 were isolated from apple juice and other types of juices and juice ingredients. Different *A.*

acidoterrestris strains demonstrate diverse growth characteristics in different types of fruit juices; some strains of *A. acidoterrestris* strains can grow well in 30% apple juice but not in 30% orange or lemon juice (37). A similar observation was also reported in our AT 4 (VF and WAC) isolates that has been first isolated and characterized by Cornell University (28). These two strains were isolated from apple juice and apple-cranberry juice, respectively, based on consumer complaints of off odors associated with these products. Both VF and WAC demonstrated different growth characteristics in the different juices; VF spores grew in citrus and tropical juices, whereas WAC spores did not. However, both strains grew well in apple juice (28). Intrinsic (pH, composition, °Brix) and extrinsic factors (storage temperature) are important parameters believed to influence the fate of the different *Alicyclobacillus* spp. strains (37). In addition to AT specific association of *Alicyclobacillus* spp. with isolation source, our results showed a random contamination pattern for the *Alicyclobacillus* spp. The same AT of *Alicyclobacillus* spp. were isolated from apple, citrus, other juices, as well as their ingredients, harvesting and processing environments, indicating a potential for contamination in many products (Table 5.2).

The most frequently isolated *Alicyclobacillus* spp. were clustered into different phylogenetic branches, directly showing the genotypic heterogeneity and taxonomic complexity of *Alicyclobacillus* spp. classification via phylogenetic analysis. Goto *et al.* (10) demonstrated the difficulties of distinguishing *A. acidocaldarius* at the subspecies level and concluded that *A. acidocaldarius* subsp. *rittmannii* DSM 11297 should be incorporated in *A. acidocaldarius*. While several strains of *A. acidocaldarius* demonstrated high DNA-DNA (92-93 %) similarity with the reference *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009, other strains revealed a minimum DNA-DNA (70-75 %) and ribotype (28-31%) similarities for being under the same

species name. This high level of genotypic heterogeneity and clustering of *A. acidoterrestris* (11) and *A. acidocaldarius* (9, 10) strains into the separate phylogenetic groups was also reported in different studies. Further studies for the improved taxonomic classification of *Alicyclobacillus* spp. are necessary.

While the focus of this study was on the most frequently isolated *Alicyclobacillus* spp., the less frequently isolated *Alicyclobacillus* spp. constituted around 13% of the all isolates with *A. fastidiosus* (9 isolates) and *A. sacchari* (5 isolates) being the most prevalent. *A. acidoterrestris* is the most commonly isolated species associated with juice spoilage, however, the potential for contamination with the less frequently isolated species of *Alicyclobacillus* should not be overlooked. These less frequently isolated *Alicyclobacillus* species may also produce guaiacol or halophenols which result in off-flavor formation (11, 20). Additional research is currently being conducted to determine whether any correlation exists between genetic relatedness and guaiacol production as well as heat resistance levels of different *Alicyclobacillus* haplotypes. To ensure the highest quality and extended shelf life, monitoring for all *Alicyclobacillus* spp. in juice production and processing environments provides more comprehensive information compared to analysis for guaiacol production alone. Juice and beverage processors benefit from a better understanding of *Alicyclobacillus* spp. prevalence and association with various juice beverages and environmental samples. This information assists in the determination of thermal processing regimes for different juice and beverage products tailored to control relevant *Alicyclobacillus* spoilage, while simultaneously avoiding excessive processing that would lead to a final product of diminished quality. Understanding the ecology of *Alicyclobacillus* spp. is beneficial for the integration of more specific non-conventional *Alicyclobacillus* spoilage controlling strategies such as high pressure (19) or via the

use of natural compounds as a means for shelf life extension and improved product quality (*1, 14, 18*).

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CHAPTER 6

CHARACTERIZATION OF *Alicyclobacillus acidoterrestris* ISOLATES FROM APPLE JUICE AND CONCENTRATES

ABSTRACT

Alicyclobacillus spp. are important spore-forming spoilage microorganisms commonly associated with off-flavor formation in apple juice and concentrates. The objective of this study was to assess the thermal resistance of and guaiacol production by *Alicyclobacillus acidoterrestris*, the most common *Alicyclobacillus* species implicated in juice spoilage.

The thermal resistance and guaiacol production levels of 18 *A. acidoterrestris* isolates from apple juice and concentrates were investigated. Additionally, one *A. acidocaldarius* strain was included in the study for comparison. Guaiacol production was monitored spectrophotometrically (490 nm) in Potato Dextrose Broth (PDB) supplemented with 100 ppm vanillic acid using the peroxidase method. Phylogenetic analysis (Paup Version 4 Beta 10) of the *A. acidoterrestris* isolates was performed using the 16S rRNA, *rpoB*, and *dnaK* sequences.

The heat resistance of *A. acidoterrestris* spores varied widely ($D_{90^{\circ}\text{C}}$ 16–59.8 min, z-values 10.4–23.5°C) in apple juice concentrate (70.9 °Brix, pH 3.4). Five strains (CU83, CU87, CU88, VF, SAC) were shown to be highly heat resistant ($D_{90^{\circ}\text{C}}$ = 59.8, 51.8, 54.3, 52.1, 52.3 min; z-values=10.4, 20.1, 13.0, 8.1, 10.8°C). All *A. acidoterrestris* strains showed detectable guaiacol levels after 5 h or 10 h of incubation in PDB (pH 3.7) at 50°C. After 48 hours, guaiacol levels ranged from

40–50 ppm for all isolates tested. Variation in guaiacol production levels was primarily due to differences in growth characteristics of the *A. acidoterrestris* strains. However, *A. acidocaldarius* strain CU92 showed low guaiacol production after 48 h at 50°C. Even though some allele types (ATs) associated groupings with guaiacol production and heat resistance of *A. acidoterrestris* were observed, more samples must be analyzed using more discriminatory genetic tools, and methods have to be explored to determine if there are any associations with specific markers that can be potentially used as indicators of spoilage potential by *A. acidoterrestris*.

Results from this study will be useful in establishing thermal processing regimes for apple juice and concentrates tailored to control relevant *A. acidoterrestris* spoilage, while avoiding excessive processing and diminished finished product quality.

INTRODUCTION

Alicyclobacillus spp. are important spore-forming bacteria commonly associated with the spoilage of various juices and beverages. The pH of most fruit juices and beverages (pH<4) had previously been thought to be too low to support the growth of spore-forming bacteria (4). Therefore, it was assumed that existing thermal processing regimes were adequate to ensure product safety during extended periods of storage at room temperature. Most bacterial spores, including *Clostridium botulinum*, cannot germinate in acidic conditions (pH < 4.6) (17). However, with the 1982 discovery of apple juice spoilage by a thermoacidophilic spore-forming bacterium (5), the juice and beverage industries became aware of the potential hazards of *Alicyclobacillus* species,

which can grow at low pH (pH 2–6.5) and at temperatures of 20–70°C (37).

Since *Alicyclobacillus* spp. are soil-borne microorganisms, their presence in raw products, surface or reclaimed processing water, and juice ingredients is unavoidable (7). Splittstoesser *et al.* (1994) isolated VF and WAC strains from apple juice and apple-cranberry juice blends from samples that were considered spoiled due to off-odors(32). The strains were later identified as *A. acidoterrestris* (26). A 1998 survey by the National Food Processors Association demonstrated that 35% of juice manufacturing companies had experienced *Alicyclobacillus* spp.-related product spoilage (35). Pettipher *et al.* (1998) found that 1 out of 4 U.S. apple juice samples and apple concentrate samples, 0 out of 56 U.K. commercial UHT fruit juice products, and 5 out of 18 U.K. fresh apple juice products were contaminated with *Alicyclobacillus* (25). Eiroa *et al.* (1999) found that 14.7% of 75 orange juice samples from 11 Brazilian producers contained *Alicyclobacillus*(9). Many acidic juices and concentrates, including apple, orange, cranberry, white grape, cherry, grapefruit, tomato, pear, mango, and pineapple, carbonated fruits drinks, and diced canned tomatoes have been found to be contaminated by *Alicyclobacillus* (5-7, 9, 15, 25, 32, 33). The isolation of *A. acidoterrestris* spores from the surface of raw fruits in their harvesting and processing environments has been demonstrated in numerous studies (7, 16, 36). Studies have indicated that not only the juice products, but also processing water, sugar, high-fructose corn syrup (HFCS), and other ingredients used in juice production could be potential sources of *Alicyclobacillus* spp. contamination (7, 21).

In the genus *Alicyclobacillus*, *A. acidoterrestris*, *A. acidocaldarius*, and *A. acidocaldarius* subsp. *acidocaldarius* are the species most frequently isolated from fruit juices and juice products (7). *A. acidoterrestris* is the major microbial spoilage

concern for the juice and beverage industries because it is widespread in the environment and leads to the production of high levels of off-odor compounds, including guaiacol, in the finished juice (18, 34). While guaiacol has a sensory detection threshold as low as 2 ppb, it has been reported that $\sim 10^5$ cells of *A. acidoterrestris* are needed to produce sufficient guaiacol to cause a detectable taint in the flavor of apple and orange juices (25). Researchers showed that all *A. acidoterrestris* strains were able to produce guaiacol (13, 37). Spoilage by *Alicyclobacillus* may occur over time and is often difficult to detect because it typically only produces off-flavor and lacks the typical gas production, turbidity, and heavy sediment associated with other microbial spoilage types. In fact, spoilage by *Alicyclobacillus* went unnoticed by juice processors until consumers started complaining (35). Consequently, Silva *et al.* (2001,2004) and Goto *et al.* (2008) suggested using *A. acidoterrestris* spores as the target microorganism during the pasteurization of juice and acidic beverages (13, 29, 30).

Conventional juice processing to achieve shelf stability is conducted at 85°C to 95°C and is designed to inactivate enzymes, pathogenic foodborne microorganisms and vegetative, non-heat resistant spoilage microorganisms (29, 32). While these thermal processes are insufficient to inactivate thermotolerant spoilage spore formers, they may activate *Alicyclobacillus* spores for germination and growth in acidic environments. Thermal processes required to inactivate heat-resistant spore formers may damage the sensory and nutritional qualities of the product and are therefore not feasible (24, 35).

To optimize pasteurization treatments for apple juice and to define precautions necessary to prevent subsequent product spoilage, it is essential to characterize the

heat resistance and guaiacol production capabilities of *A. acidoterrestris* strains. In this study, the heat resistance and guaiacol production of 18 selected *A. acidoterrestris* strains and one *A. acidocaldarius* strain isolated from apple juice, apple juice concentrates, or apple cranberry juice were characterized. In addition, interactions of 16s rRNA, *dnaK*, *rpoB* gene-based allelic type (AT) groupings with heat resistance and guaiacol production were investigated.

MATERIALS AND METHODS

Spore preparation. *A. acidoterrestris* and *A. acidocaldarius* strains were grown in Potato Dextrose Agar (PDA) (pH 3.5) and incubated at 50°C for 7–10 days. Spores were collected by scraping colonies from the agar surface using 2–3 mL deionized sterile water. Harvested spores were washed with sterile deionized water 3 times; spore samples were centrifuged (13,800 g, 5 min) after each washing. After the final washing, spores were suspended in 300 µL deionized water and stored at -80°C. On the day of the experiment, the entire spore population was added to 10 mL of apple juice concentrate (70.9 °Brix, pH 3.4) and a suspension of 10^6 – 10^7 spores/mL was prepared. Pasteurized apple juice clear concentrate was obtained from Invertec Foods (Vitacura, Santiago, Chile). Brix was measured using an Auto Abbé refractometer 10500 (Leica Inc., Buffalo, NY) and pH values were measured using a Thermo Scientific Orion 2-Star pH meter (Waltham, MA).

Isolation of acidothermophilic sporeformers. Acidothermophilic spore-forming *Alicyclobacillus* spp. were isolated from apple juice, apple juice blends, and apple juice concentrates by membrane filtration, media plating, and enrichment process as previously described (7).

Decimal reduction times and z-values. Thermal resistance of *A. acidoterrestris* and *A. acidocaldarius* spores was determined using 1.7×100 mm glass capillary tubes as described by Splittstoesser *et al.* (1996). Decimal reduction times (D-values) for 90°C, 95°C, and 98°C were calculated from the slope of the regression line of the spore survival curves, while z-values were calculated from the regression line of the D-values versus temperature curve.

Guaiacol detection. Guaiacol production was assessed using vegetative cells of *A. acidoterrestris*. Cells were obtained from PDA plates (pH: 3.5) incubated at 50°C for 18–24 h. A single colony from each plate was retrieved using a sterile loop, transferred to a sterile centrifuge tube with 1.0 mL sterile water, and the contents vortexed. Ten µL of the resulting bacterial cell suspension were added to 10 mL Potato Dextrose Broth (PDB, Difco laboratories, Detroit, Michigan) supplemented with 100 ppm vanillic acid (98.5+%, Acros Organics, Springfield, NJ) in a screw-capped test tube. The initial concentration of the vegetative cells was ranged between 10^1 to 10^2 CFU/mL and cell counts were monitored over time.

Guaiacol production was monitored over 5, 10, 24, and 48 h of incubation at 50°C. Each assay was performed at least in triplicate and values were averaged. A spectrophotometric guaiacol detection method was adopted based on the peroxidase guaiacol detection method described by Niwa *et al.* (2003) (22). Briefly, 200 µL of PDB (pH 3.7) were added to each well of a 96-well flat-bottom microtiter plate (Costar, Cambridge, MA) from the test and control tubes. Then, 100 µL of 33 mM potassium hydrogen phthalate buffer ($\geq 99\%$, Sigma-Aldrich, St Louis, MO) and 2 µL

1.3% H₂O₂ were added to each well. Finally, 2 µL (0.486 unit/well) of peroxidase (Type II from HRP, Sigma-Aldrich, St Louis, MO) was added to each well. Measurement of guaiacol concentration was performed at 490 nm using a 96-well multiscanner (Dynex Technologies, MRX-TC Revelation, Chantilly, VA). A calibration curve was generated using known amounts of guaiacol (99%, Sigma-Aldrich) in PDB (Figure 6.1).

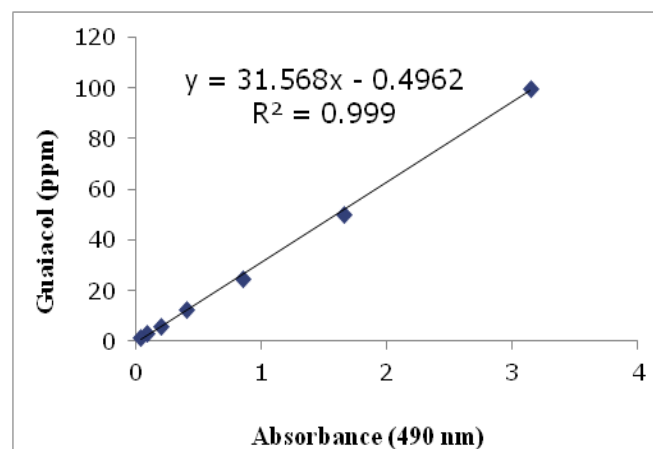


Figure 6.1. Standard curve for guaiacol measurement using a 96-well plate modified peroxidase method

16S rRNA, *dnaK*, and *rpoB* haplotype analysis. Gene sequences (16s rRNA, *dnaK*, *rpoB*) for all test strains were determined using a MegAlign[®] (DNASTar[®], Lasergene) DNA sequencer. Descriptive analyses, including allele assignment, were performed using DnaSP version 5.0 (Sinauer Associates, Inc., Sutherland, MA). Different alleles were assigned to gene sequences that differed from each other at one or more nucleotide sites. Isolates with 2 different allelic types were considered to represent 2 different molecular subtypes. Consensus trees were constructed using the neighbor-joining (NJ) method with 2000 bootstrap replicates for the 16S rRNA, *dnaK*, and *rpoB*

gene sequences of *A. acidoterrestris* and *A. acidocaldarius* isolates. *A. acidoterrestris* ATCC 49025 (GenBank accession number AB042057), *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009 (GenBank accession number AB042056), *A. acidocaldarius* ATCC 43030 (GenBank accession number AY573796), and *Alicyclobacillus* genomic species 1 DSM 11984 (GenBank accession number AB059668) nucleotide sequences were included in the 16S rRNA phylogenetic tree as reference strains while the *A. hesperidum* DSM 11985 (GenBank accession number AB291060) nucleotide sequence was used as an outgroup in the analysis. Because *dnaK* and *rpoB* nucleotide sequence data for *Alicyclobacillus* spp. were unavailable in the National Center for Biotechnology Information (NCBI), reference strains could not be included in the *dnaK* and *rpoB* consensus trees.

Primers design and PCR conditions. A hypervariable region (HV) of the 16S rRNA gene of *Alicyclobacillus* strains was amplified by polymerase chain reaction (PCR) using universal forward (5'-AGAGTTTGATCCTGGCTCAG-3') (Weisburg *et al.*, 1991) and reverse (5'-GGTATCTAATCCTGTTTGC-3') primers, as previously described (7). PCR primers for amplification of *rpoB* and *dnaK* were designed using Primer Select[®] (DNASTar[®], Lasergene, WI) based on alignment of *Bacillus* spp., *Geobacillus* spp., and *Paenibacillus* spp. DNA sequences available for the respective genes in GenBank. Alignments of 7 *rpoB* (GenBank accession numbers DQ642058, EF015348, EF015360, EF015387, EF015390, EU137649, and CP000813) were used to create an *rpoB* forward primer (5'-GCGAAGTGTTAGAATTACC-3') and a reverse primer (5'-TCGTATTCTAACCATGCGCC-3'). A set of degenerate *dnaK* primers [forward primer (5'-CARGCNCANAARGAYGCNGG-3'); reverse primer (5'-GCNACNGCYTCRTCNGGR-3')] were created from 7 *dnaK* GenBank sequences (Accession numbers ABDK02000006, ABDK02000011, ABVH01000003,

AE017333, CP000002, NC006270, and NZABCD02000006).

The preparation of DNA lysates and amplification of 16S rRNA gene fragments were performed as previously described (7). A touchdown PCR program was used for both *rpoB* and *dnaK* gene fragment amplifications. The thermocycler program for the *rpoB* gene was as follows: denaturation at 95°C for 15 min followed by 20 cycles of 94°C for 30 sec; annealing at 60°C for 30 sec; and extension at 72° for 1 min. The annealing temperature was decreased by 0.5°C per cycle. This was followed by 20 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The same touchdown PCR program was used for the *dnaK* primers except the initial annealing temperature was 50°C for the first 20 cycles and 40°C for the following 20 cycles. PCR DNA was purified as described previously (7).

Simpson index of discrimination (SID). Simpson's index of diversity (SID) with 95% confidence intervals was calculated as described by Hunter and Gaston (1988).

RESULTS

Cluster analysis of 16S rRNA, *dnaK*, and *rpoB* genes. A total of 18 *A. acidoterrestris* strains and one *A. acidocaldarius* strain were chosen from previously-isolated strains for 16S rRNA, *dnaK* and *rpoB* sequence subtyping. Eleven 16S rRNA, 4 *dnaK*, and 7 *rpoB* allelic types (ATs) were differentiated among the isolates (Table 1; Figures 6.2, 6.3, and 6.4).

Table 6.1. Heat resistance, guaiacol production, and haplotype assignments for *A. acidoterrestris* isolates.

Strains	Species ID	D- value (min)				Allelic Types (ATs)			Guaiacol Production (ppm)			
		90°C	95°C	98°C	z-value (°C)	16s	<i>dnaK</i>	<i>rpoB</i>	5 h	10 h	24 h	48 h
CU80	<i>A. acidoterrestris</i>	22.5	14.7	6.3	15.2	1	1	4	0.5	3.6	43.2	40.5
CU81	<i>A. acidoterrestris</i>	22.4	11.8	8.6	19.1	2	2	2	8.3	36.0	47.1	46.4
CU82	<i>A. acidoterrestris</i>	20.1	13.6	5.0	14.0	1	1	1	4.4	21.1	43.6	41.2
CU83	<i>A. acidoterrestris</i>	59.8	31.8	9.4	10.4	1	1	2	4.4	26.6	42.4	38.6
CU84	<i>A. acidoterrestris</i>	27.2	10.2	6.0	12.2	1	1	3	3.2	24.1	46.1	47.0
CU85	<i>A. acidoterrestris</i>	20.1	13.0	5.0	13.9	1	1	1	2.0	6.2	46.6	45.9
CU86	<i>A. acidoterrestris</i>	32.8	26.6	4.6	10.2	3	3	2	13.3	38.1	48.7	46.1
CU87	<i>A. acidoterrestris</i>	51.8	37.1	19.9	20.1	4	2	2	10.4	39.3	44.6	44.2
CU88	<i>A. acidoterrestris</i>	54.3	20.7	13.3	13.0	5	2	1	21.3	38.3	45.5	49.8
CU89	<i>A. acidoterrestris</i>	23.6	9.4	7.1	15.0	6	2	2	5.6	38.7	43.3	45.5
CU90	<i>A. acidoterrestris</i>	16.0	8.3	4.6	15.0	7	2	2	7.8	38.8	45.2	43.8
CU91	<i>A. acidoterrestris</i>	25.9	16.0	5.1	11.9	5	2	2	9.1	41.1	44.2	44.1
CU93	<i>A. acidoterrestris</i>	28.4	16.4	6.3	12.8	1	1	5	-0.3	1.6	40.2	44.4
VF	<i>A. acidoterrestris</i>	52.1	15.9	5.2	8.1	3	3	1	3.2	30.3	39.0	45.0
WAC	<i>A. acidoterrestris</i>	41.3	15.8	5.3	9.2	11	3	1	4.7	30.0	44.1	43.7
SAC	<i>A. acidoterrestris</i>	52.3	25.2	9.0	10.8	10	2	7	7.9	39.7	46.5	48.2
IPC	<i>A. acidoterrestris</i>	30.6	16.7	11.3	18.6	3	3	1	11.0	38.8	39.0	44.0
KF	<i>A. acidoterrestris</i>	40.5	20.5	6.1	10.2	9	1	6	7.6	37.1	44.8	45.8
CU92	<i>A. acidocaldarius</i>	29.3	26.3	10.0	18.7	8	4	2	-0.7	-0.8	-0.5	5.6

An alignment of the 656 base pair (bp) 16S rRNA gene sequence (corresponding nucleotides 71 to 726 of the 1514 bp 16s rRNA ORF gene from *A. acidoterrestris* [GenBank AB059675]) for all *Alicyclobacillus* species was conducted and a phylogenetic tree was generated (Figure 6.2). The variable region of the 16s RNA gene sequencing differentiated 11 ATs (Table 6.1).

A. acidoterrestris strains were grouped into 2 lineages (Lineage 2 and Lineage 3) while the *A. acidocaldarius* (CU92) strain was grouped in a separate lineage (Lineage 1) along with ATCC 27009 (*A. acidocaldarius* subsp. *acidocaldarius*; AB042056), ATCC 43030 (*A. acidocaldarius*; AY573796), and *Alicyclobacillus* genomic species 1 DSM 11984; AB059668 (Figure 6.2). While AT 1 consisted of 6 *A. acidoterrestris* strains, AT5 and AT3 and had 2 and 3 strains, respectively. In addition, strain CU92 and *Alicyclobacillus* genomic species 1 DSM 11984 AB059668 shared a unique AT.

Amplified fragments of *dnaK* (491 bp) and *rpoB* (414 bp) genes were more conserved among the *A. acidoterrestris* strains and demonstrated a lower genetic diversity and subtype discrimination than the 16s rRNA gene. The *dnaK* NJ gene, with 3 different lineages and 4 different ATs, had the least subtyping discrimination among the genes analysed. However, all 18 *A. acidoterrestris* strains were grouped in 3 different ATs.

While all lineage 2 strains were grouped under AT 1 (7 strains), lineage 3 strains were grouped under AT 2 (7 strains) and AT 3 (4 strains). *A. acidocaldarius* was grouped under AT 4 (Figure 6.3). The amplified region of *rpoB* gene resulted in 7 unique ATs. Most of the isolated genes were grouped in AT 1 (8 strains) and AT 2 (6 strains) (Figure 6.4). SID showed a higher diversity for 16S rRNA (SID=0.889) than for *dnaK* (SID=0.72) and *rpoB* (SID=0.75) ATs.

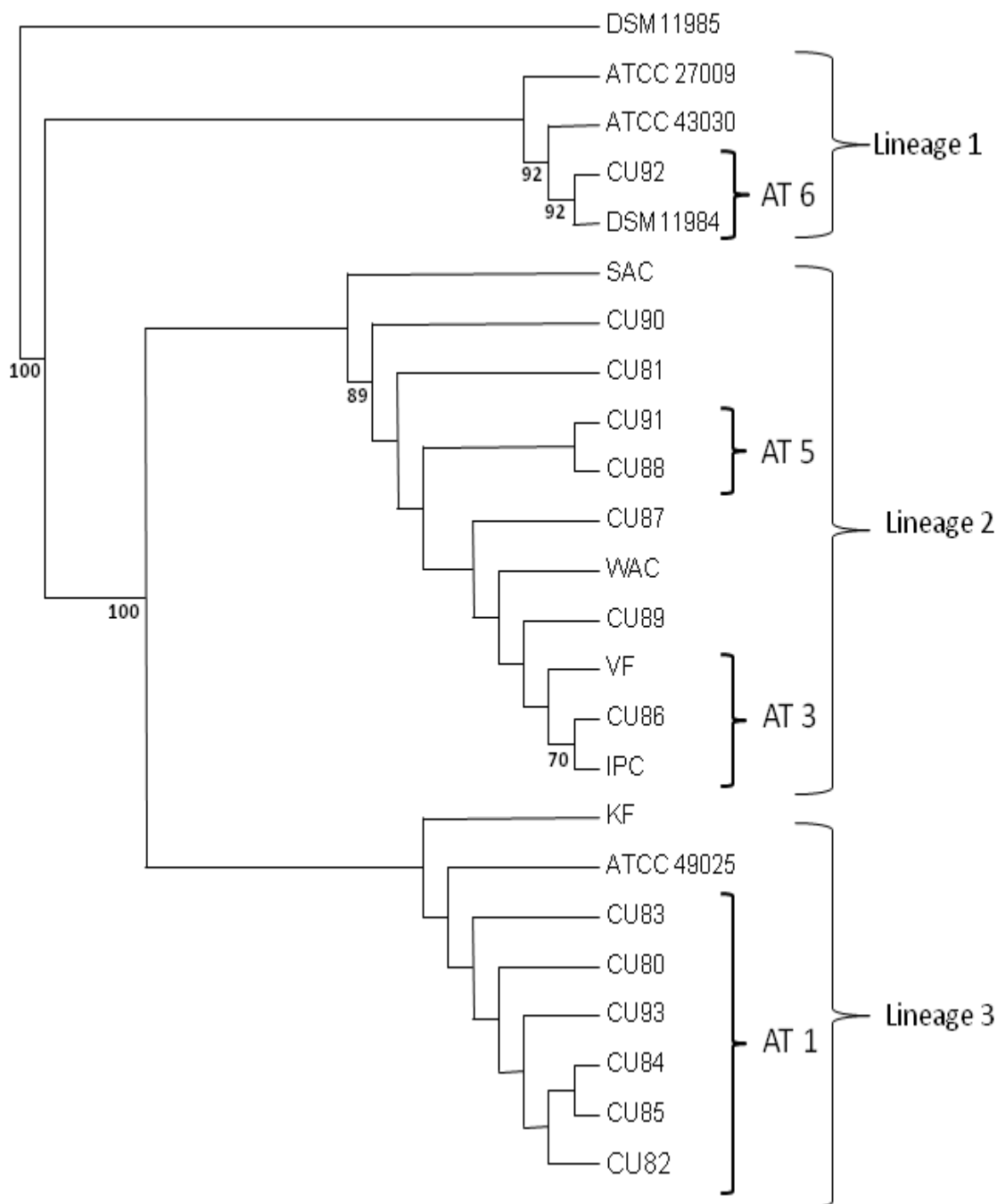


Figure 6.2. 16S rRNA gene-based consensus tree for 18 *A. acidoterrestris* isolates and one *A. acidocaldarius* isolate. The tree was constructed using the Neighbor-Joining method with a 656 bp including hypervariable region of the 16S rRNA fragments. *A. acidoterrestris* ATCC 49025 (AB042057), *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009 (AB042056), *A. acidocaldarius* ATCC 43030 (AY573796), *Alicyclobacillus* genomic species 1 DSM 11984 (AB059668), *A. hesperidum* DSM 11985 (AB291060) nucleotide sequence were included as reference strains. A total of 2000 bootstrap replications were performed (frequencies <70 % are not shown).

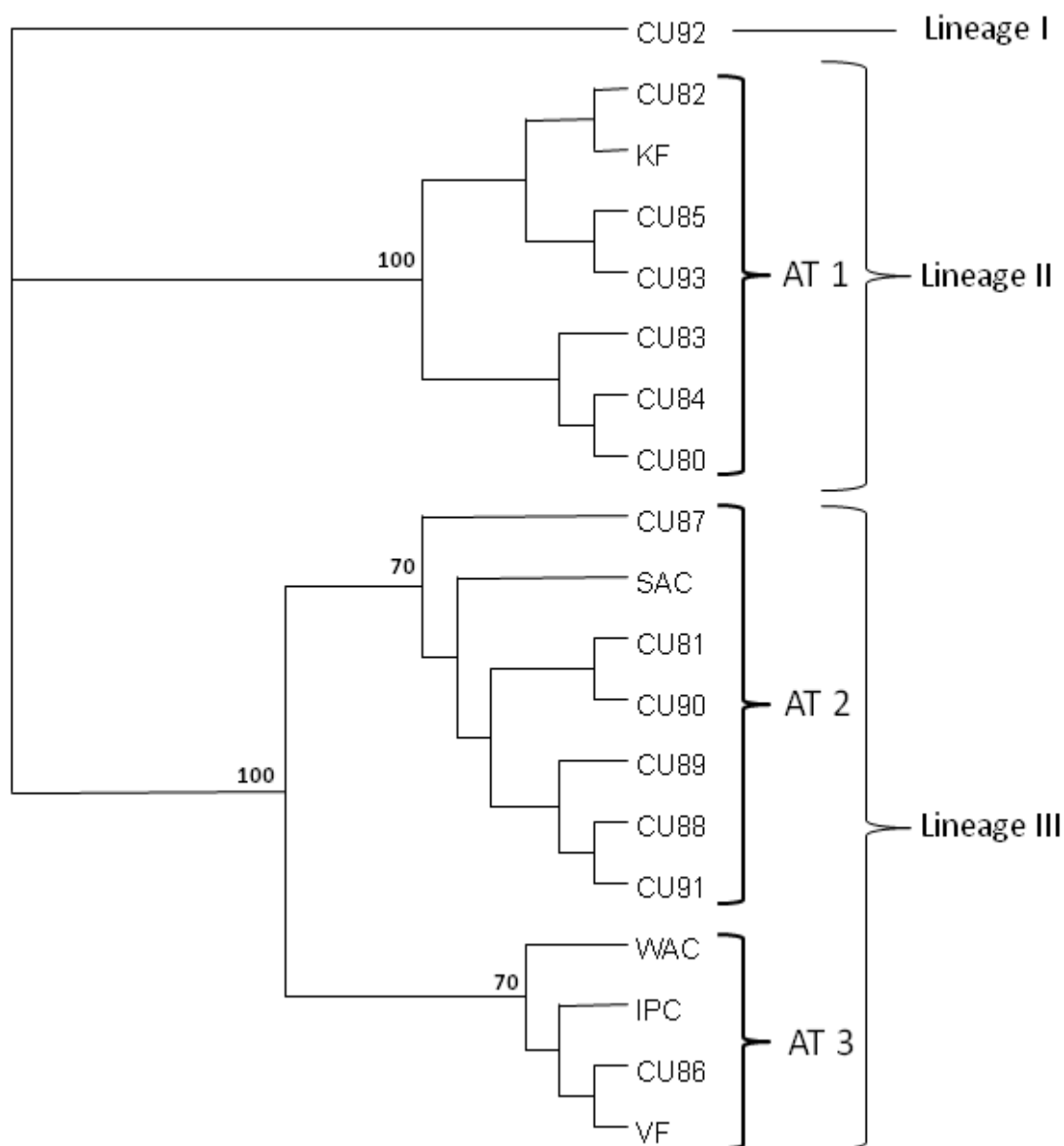


Figure 6.3. *dnaK* gene-based consensus tree for 18 *A. acidoterrestris* isolates and one *A. acidocaldarius* isolate constructed using the Neighbor-Joining method with a 491 nucleotide sequence of *dnaK* fragments. A total of 2000 bootstrap replications were performed (frequencies <70 % are not shown)

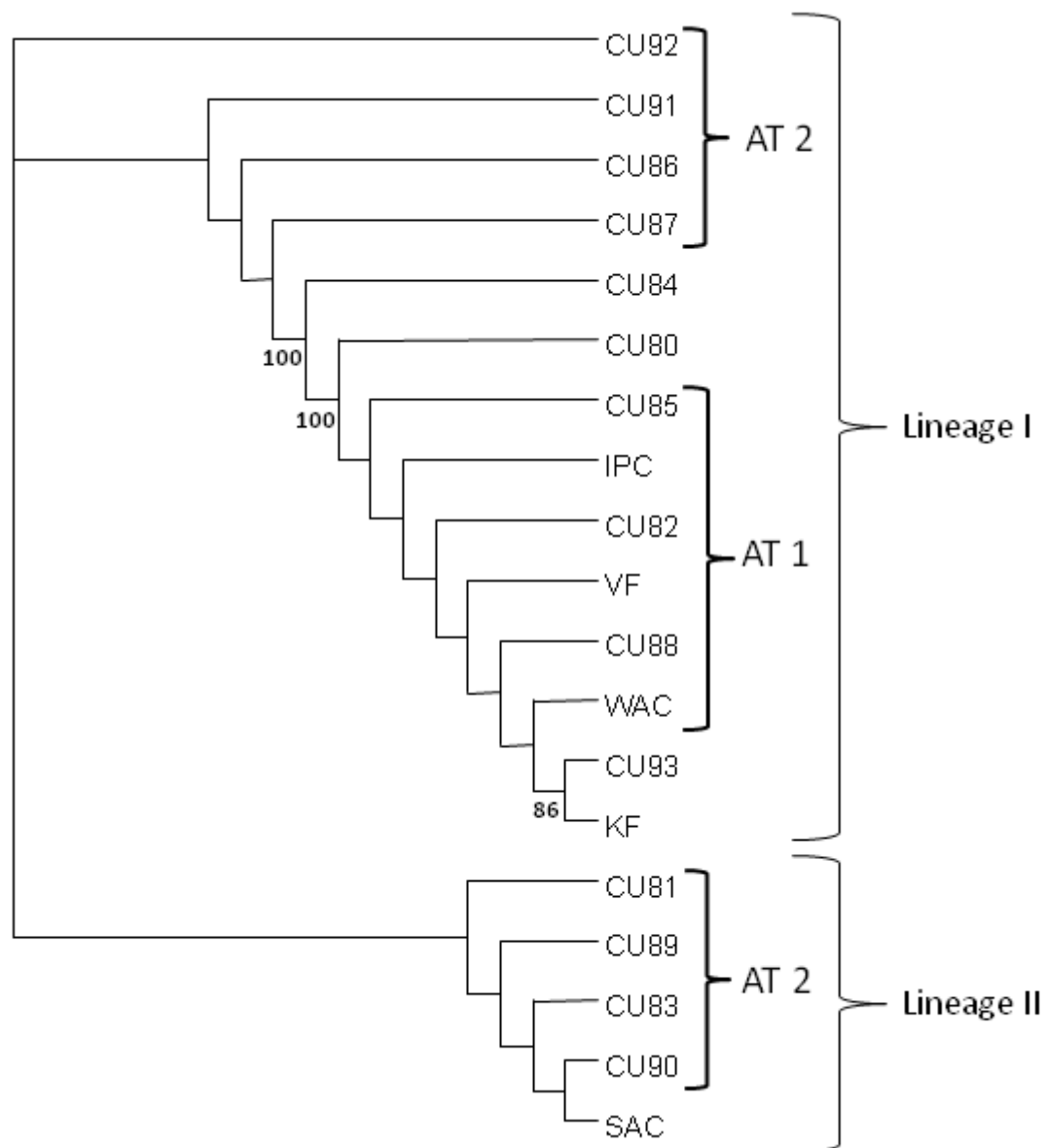


Figure 6.4. *rpoB* gene-based consensus tree for 18 *A. acidoterrestris* isolates and one *A. acidocaldarius* isolate constructed using the Neighbor-Joining method with a 414 nucleotide of the *rpoB* fragments. A total of 2000 bootstrap replications were performed (frequencies <70 % are not shown)

Guaiacol production. All *A. acidoterrestris* strains demonstrated high levels (40–50 ppm) of guaiacol production after 24 h incubation at 50°C in PDB (pH 3.7) supplemented with 100 ppm vanillic acid. *A. acidoterrestris* isolates which were identified as AT 1 according to 16S rRNA and *dnaK* genes haplotype analysis seemed to be grouped according to their guaiacol production concentrations (ppm) at hour 10 of analysis (Figures 6.5 and 6.6). Guaiacol production rate varied among the different ATs of *A. acidoterrestris* strains at 5 h and 10 h of analysis. However, since all *A. acidoterrestris* strains were able produce 40–50 ppm guaiacol after 24 and 48 h, overall, there were no differences in guaiacol production of different ATs. Interestingly, the majority of *dnaK* AT 1 strains were grouped at a lower level than *dnaK* AT 2 strains at 5 h and 10 h of analysis (Figure 6.6). With respect to the *rpoB* ATs, there was no clear separation at any hour of analysis of guaiacol production. However, the *A. acidocaldarius* (CU92) strain required 48 h incubation at 50°C in PDB (pH 3.7) to produce a detectable amount of guaiacol. *A. acidocaldarius* CU92 strain (16S rRNA AT 8, *dnaK* AT 4, *rpoB* AT 2) was included at the bottom level of 16S rRNA, *dnaK*, and *rpoB* 2D-scatter plot analysis (Figures 6.5, 6.6, and 6.7).

Heat resistance. The heat resistance of *A. acidoterrestris* spores varied widely ($D_{90^{\circ}\text{C}}$ 16.0–59.8 min; z-values 10.4–23.5°C) in apple juice concentrate (70.9°Brix, pH 3.4) (Table 6.1). Five strains (CU81, CU85, CU86, VF, SAC) were shown to be highly heat resistant ($D_{90^{\circ}\text{C}}$ =59.8, 51.8, 54.3, and 52.1 min; $D_{95^{\circ}\text{C}}$ =31.8, 37.1, 20.7, 15.9, and 25.2 min; $D_{98^{\circ}\text{C}}$ =10.4, 20.1, 13.0, 8.1, and 10.8 min; z-values=10.4, 20.1, 13.0, 8.1, and 10.8°C, respectively) (Table 6.1). While AT1 subtypes of 16S rRNA and *dnaK* genes had a clustering between 20 and 30 min for $D_{90^{\circ}\text{C}}$ -values, there were no obvious correlations between the heat resistance of strains and various ATs (Figures 6.8, 6.9, and 6.10).

DISCUSSION

The results of this study demonstrated that all 18 *A. acidoterrestris* strains previously isolated from apple juice, apple-cranberry juice, and apple juice concentrates can produce high amounts of guaiacol (40–50 ppm) in PDB (pH 3.7) supplemented with 1% vanillic acid after 24 h incubation at 50°C, although the amounts of guaiacol produced varied over time among the different *A. acidoterrestris* strains. In this study, *A. acidoterrestris* strains CU86, CU87, CU88, IPC produced >10 ppm of guaiacol after 5 h of incubation, while *A. acidoterrestris* strain CU93 and *A. acidocaldarius* strain CU92 had undetectable guaiacol production after 5 h of incubation. Goto *et al.* (2008) categorized selected *Alicyclobacillus* strains into three groups based on their guaiacol production rates in 5 hours in Va-YSG broth at 45°C : group I <10 ppm, group II <10 ppm, and group III >10 ppm (13). However, all strains demonstrated guaiacol production of 40–50 ppm after 48 h of incubation (13). Guaiacol is easily produced by *A. acidoterrestris* during ferulic acid metabolism in vanillic acid-supplemented media, but the mechanisms of guaiacol formation in juice are not fully understood (31). While vanillic acid could be present in the juice because of contamination, it could be also be naturally derived from the plant polymer lignin. Tyrosine, present at concentrations of approximately 4.1 µg/mL in apple juice and 3.4–13.5 µg/mL in orange juice, may also be a precursor to guaiacol formation in juices (6). *A. acidoterrestris* is known to be the major producer of guaiacol in juice products and is the major microbial spoilage concern for the juice and beverage industries because it is widespread in the environment. It is responsible for the production of off-odor compounds, including guaiacol (34). While *A. acidiphilus*, *A. herbarius*, and *A. hesperidum* subs. *aigle* also produce guaiacol, their association with juice spoilage is rare (13).

Komitopoulou *et al.* (1999) subjectively detected guaiacol in apple juice and orange juice when the population of *A. acidoterrestris* strain Z CRA 7182 reached 10^5 CFU/mL after juice storage at 30°C for 4 days and 25°C for 8 days (19). In addition, the same strain produced detectable guaiacol in grapefruit juice at 10^4 CFU/mL. Similarly, Pettipher *et al.* (1997) reported that 10^5 CFU/mL of *A. acidoterrestris* strain 2498 was required before the microorganism produced sufficient guaiacol (about 2 ppb) to cause a detectable taint in products (25). Orr *et al.* (2000) demonstrated that growth of *A. acidoterrestris* strains (N-1096, N-1100, N-1108, OS-CAJ, SAC) in apple juice was very slow because the number of spores and vegetative cells of *A. acidoterrestris* remained constant during 61 days of storage at 21°C or 37°C period. However, a sensory panel detected guaiacol in 5 samples stored for 8–61 days even though guaiacol was not detected using gas chromatography (23). These results and findings from the current study suggest that guaiacol production varies with the bacterial strain, the composition of the growth medium, and temperature. Guaiacol production by *A. acidoterrestris* is also dependent on the concentration of germinated spores in the juice, strain type, headspace oxygen concentration, storage conditions, thermal pasteurization parameters, and the concentration of guaiacol precursors present (2, 13). Identification of high-guaiacol-producing *A. acidoterrestris* strains would help in the development of control strategies. It should be noted that the sensory detection threshold for guaiacol is 2 ppb, which is much lower than the detection limit achieved using a spectrophotometric method in this study (~1.5 ppm). A more sophisticated method, such as gas chromatography/mass spectrometry (GC/MS), is needed to achieve a lower guaiacol detection level.

In this study, *A. acidoterrestris* spores showed wide variability in heat resistance ($D_{90^{\circ}\text{C}}$ 16–59.8 min; z-values 10.4–23.5°C) in apple juice concentrate (70.9°Brix, pH 3.4). This study also determined the heat resistance of lab-isolated VF (apple juice isolate) and WAC (apple-cranberry juice blend) strains (Table 6.1). Splittstoesser *et al.* (1994) determined the heat resistance of an *A. acidoterrestris* VF strain in apple juice (pH 3.5, 11.4°Brix) and WAC strain in grape juice (pH 3.3, 11.4°Brix). $D_{90^{\circ}\text{C}}$ -values for the VF and WAC strains were 23 min and 16 min, respectively, while $D_{95^{\circ}\text{C}}$ - values were 2.8 min and 2.4 min, respectively (32). Pontius *et al.* (1998) also tested *A. acidoterrestris* VF and WAC strains in buffers designed to represent a model fruit juice (12% glucose and 30 mM malic acid; °Brix not reported). The $D_{91^{\circ}\text{C}}$ -values for VF and WAC were 54.3 min and 53.2 min, respectively, and $D_{97^{\circ}\text{C}}$ -values were 8.8 and 9.0, respectively, at pH 3.7. Both strains had reported z-values of 7.7°C (26). Differences in D-values for the isolates in these studies and the current study are due principally to the difference in the soluble solids content of the test environment because increasing levels of soluble solids are known to increase D-values. For example, Splittstoesser *et al.* (1998) found that the $D_{90^{\circ}\text{C}}$ value of *A. acidoterrestris* strain WAC increased from 11 to 18 min and the $D_{95^{\circ}\text{C}}$ value increased from 1.9 to 2.3 min as the soluble solids increased from 16 to 30 °Brix in Concord grape juice. When the °Brix was increased from 30 to 65, the $D_{90^{\circ}\text{C}}$ -value increased from 18 to 127 min and the $D_{95^{\circ}\text{C}}$ value increased from 2.3 to 12 min (32). The thermal heat resistance of *A. acidoterrestris* has been shown to be affected most by temperature (85-97°C), followed by the soluble solids content (5-60°Brix), and pH (2.5-6.0) of juice and juice beverages (3, 20, 28). Similarly, Silva *et al.* (1999) showed that the $D_{91^{\circ}\text{C}}$ -values of *A. acidoterrestris* (NCIMB 13137) spores increased from 3.8 to 24.1 min as the soluble solids level of blackcurrant concentrate increased from 26.1 to 58.5°Brix (28).

Heat resistance of *A. acidoterrestris* spores is affected by many factors, including sporulation conditions (*e.g.*, sporulation medium, temperature), the physical state of the microorganism (*e.g.*, heat-induced resistance after heat activation of spores, sporulating cells), composition of the heating medium (*e.g.*, pH, °Brix), and the recovery condition of heat treated bacterial spores. Therefore, data can only be compared within the same study or among studies that have used comparable methodology (27). The reported z-values in Table 6.1 would be a valuable tool when considering changes to commercial juice pasteurization temperatures. Increasing the processing temperature would increase product safety; decreasing the temperature would preserve the quality of the juice (3).

16S rRNA gene-based subtyping is generally considered to have limited utility due to the relatively low gene diversity within most bacterial species (8, 10). However, Goto *et al.* (2002) demonstrated that the 16S rRNA nucleic acid sequencing includes a hypervariable (HV) region at the 5' end (nt 70 to 344) among *Bacillus* (14) and *Alicyclobacillus* species (11). SID analysis also showed a higher diversity for 16S rRNA (SID=0.889) than for *dnaK* (SID=0.72) and *rpoB* (SID=0.75) ATs, indicating that amplification of the 16S rRNA hypervariable (HV) region at the 5' end (nt 70 to 344) is a useful index for the identification, distribution, and grouping of *Alicyclobacillus* species (11). In contrast, 16S rRNA gene sequence information cannot be relied on for identification of *Alicyclobacillus* species using similarity searches against the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) due to high genotypic heterogeneity and taxonomic complexity in the *Alicyclobacillus* genus. Goto *et al.* (2006) indicated that *A. acidocaldarius* is indistinguishable at the subspecies level according to their phenotypic, chemotaxonomic, genotypic, and phylogenetic features and concluded that

A. acidocaldarius subsp. *rittmannii* DSM 11297 should be included in *A. acidocaldarius* (12).

While the CU92 strain used in the current study was closely clustered with *A. acidocaldarius* and *A. acidocaldarius* subsp. *acidocaldarius* strains in the 16s rRNA analysis using 141 isolates of *Alicyclobacillus* isolates (7), the CU92 strain shared the same 16s rRNA AT 6 with *Alicyclobacillus* genomic species 1 DSM 11984, which should be listed under *Alicyclobacillus* genomic species 1. Since biochemical, physiological, and chemotaxonomic characteristics of *Alicyclobacillus* genomic species 1 DSM 11984 are indistinguishable from those of *A. acidocaldarius* strains, further extensive phylogenetic analysis would increase understanding of strain relatedness (1). Since both *rpoB* and *dnaK* degenerate primers were designed from homologous sequence regions of closely related *Bacillus* spp., *Geobacillus* spp., and *Paenibacillus* spp., their subtyping abilities were limited. In addition, simpson index diversity (SID) measurement results confirmed that the amplified regions of both *dnaK* (SID: 0.72) and *rpoB* (SID: 0.75) genes had a lower discriminatory power over 16S rRNA (SID: 0.889) gene. More discriminatory subtyping methods need to be developed for further genetic characterization of *Alicyclobacillus* species.

Even though some AT-associated groupings with guaiacol production and heat resistance of *A. acidoterrestris* were observed, more samples must be analyzed using more discriminatory genetic tools and methods. Apple juice and beverage processors will benefit from the improved characterization and monitoring of the *A. acidoterrestris* strains isolated from apple juice and concentrates. This information will be valuable in establishing thermal processing regimes for apple juice and concentrates that will provide protection against *Alicyclobacillus* spoilage while

retaining product quality.

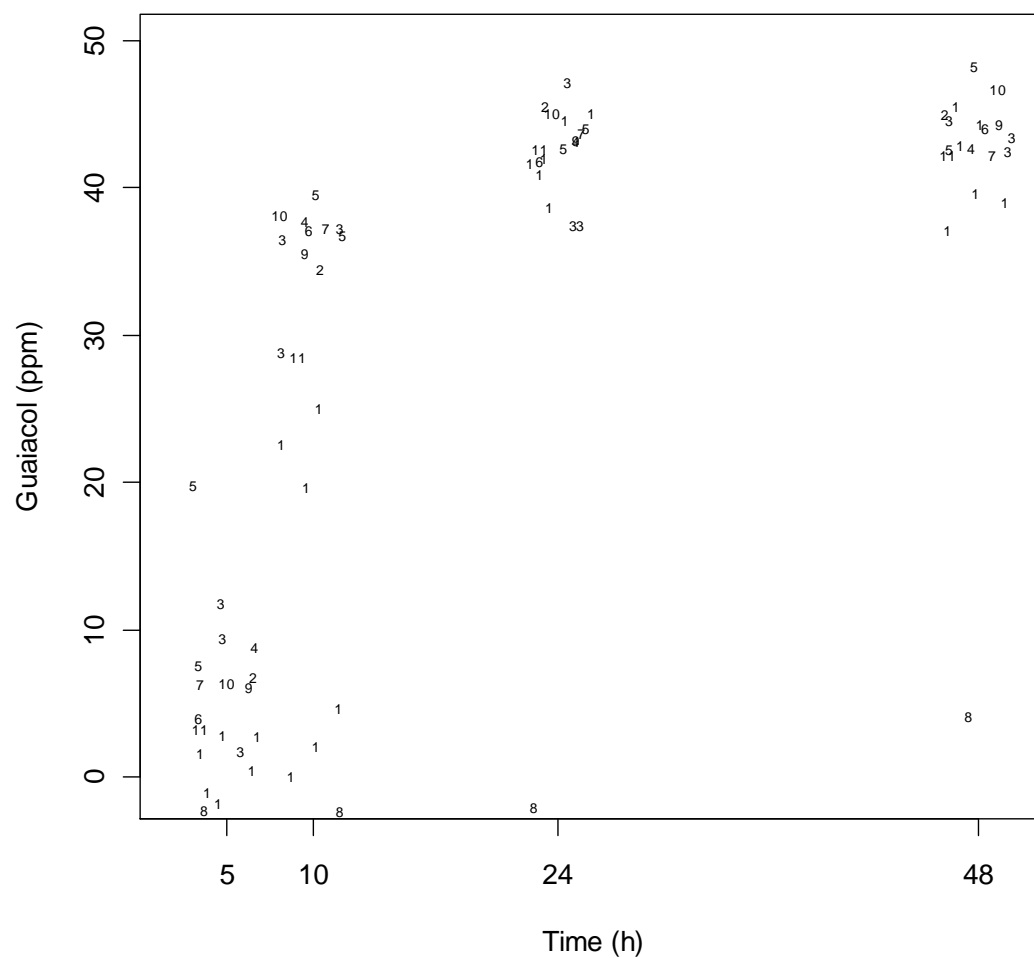


Figure 6.5. Scatter plot showing interaction between 16s ATs groupings and guaiacol production of *A. acidoterrestris* and *A. acidocaldarius* strains.

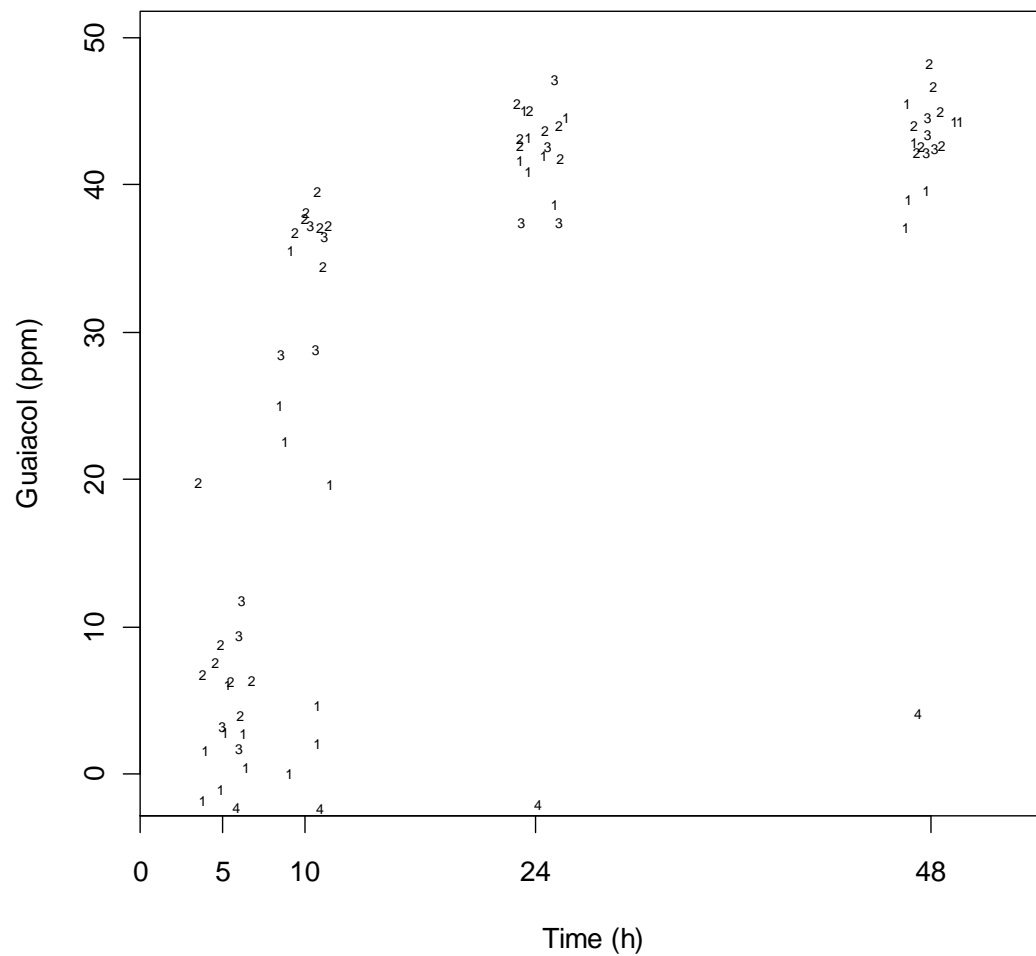


Figure 6.6. Scatter plot showing interaction between *dnaK* ATs groupings and guaiacol production of *A. acidoterrestris* and *A. acidocaldarius* strains

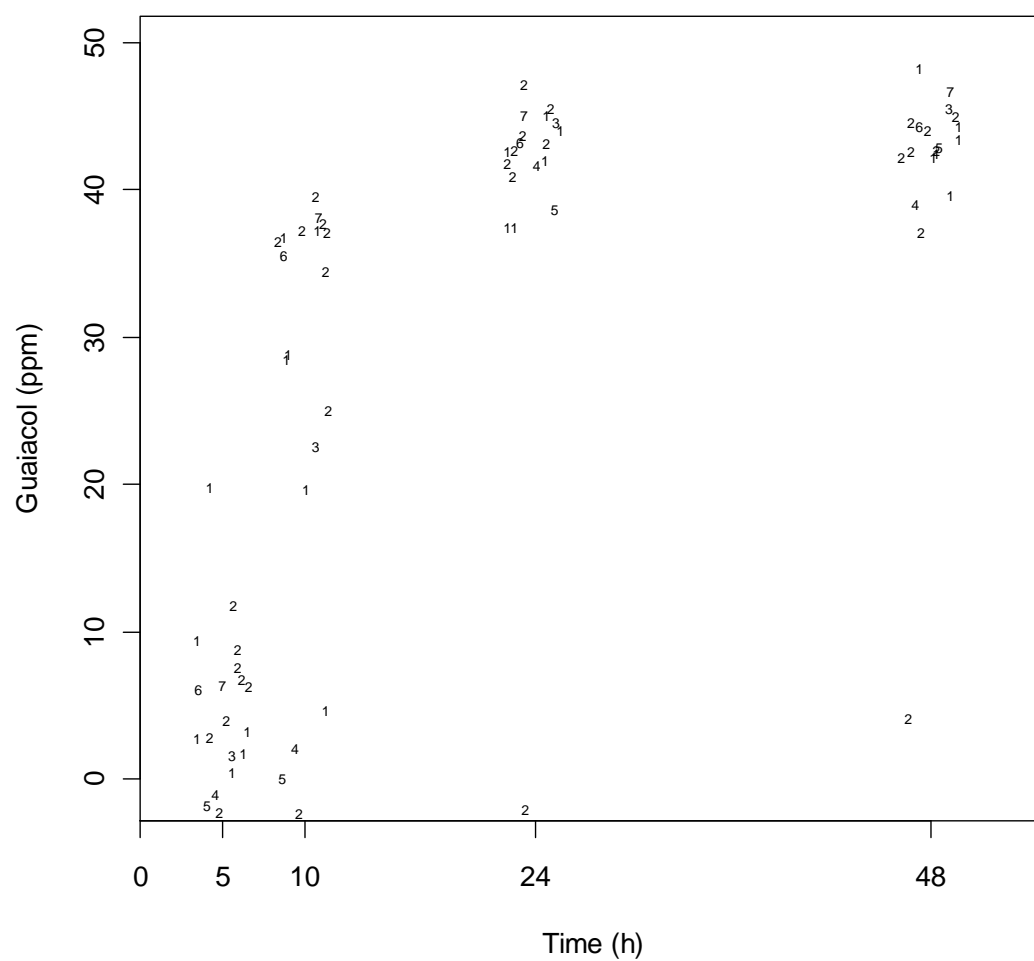


Figure 6.7. Scatter plot showing interaction between *rpoB* ATs groupings and guaiacol production of *A. acidoterrestris* and *A. acidocaldarius* strains

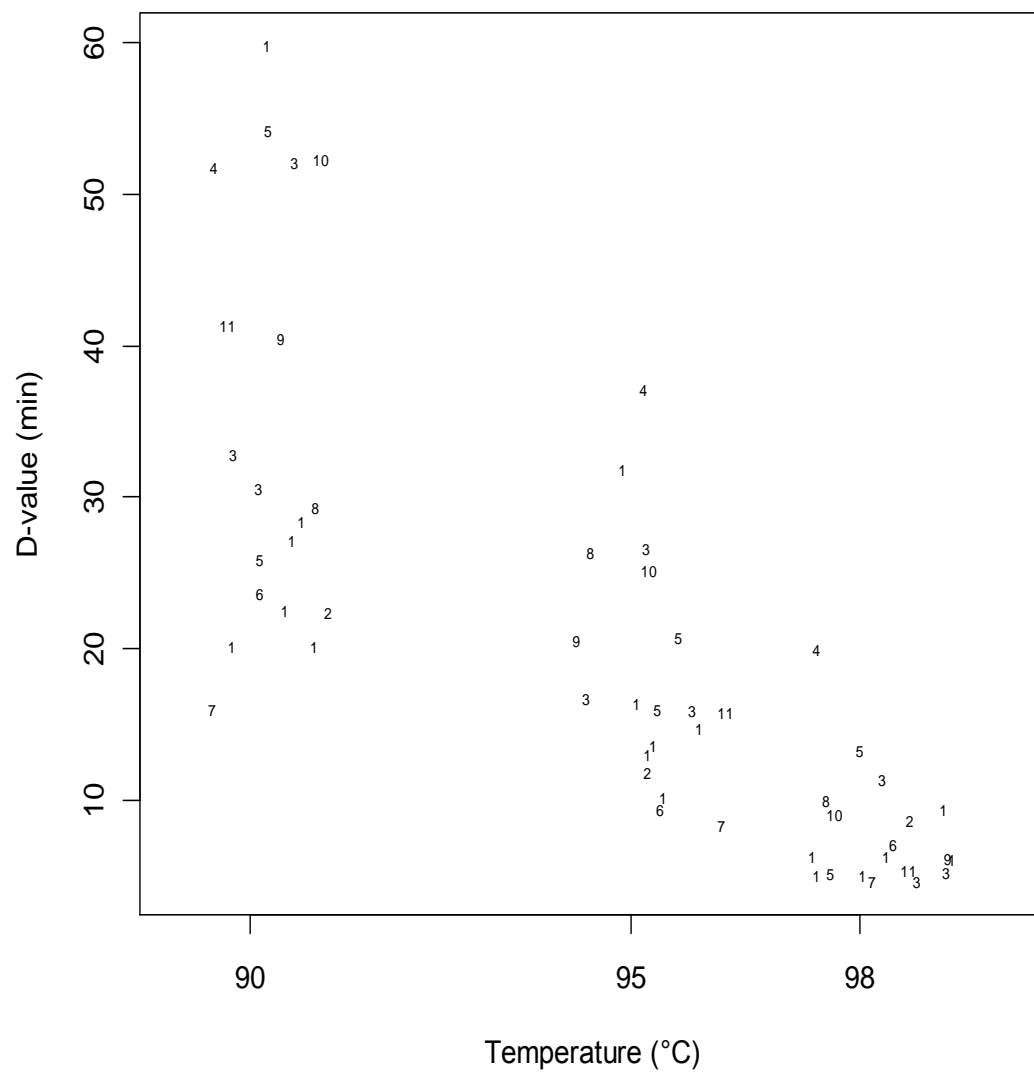


Figure 6.8. Scatter plot showing groupings of 16s ATs and heat resistance (D_{90} , D_{95} , and D_{98}) of *A. acidoterrestris* and *A. acidocaldarius* strains

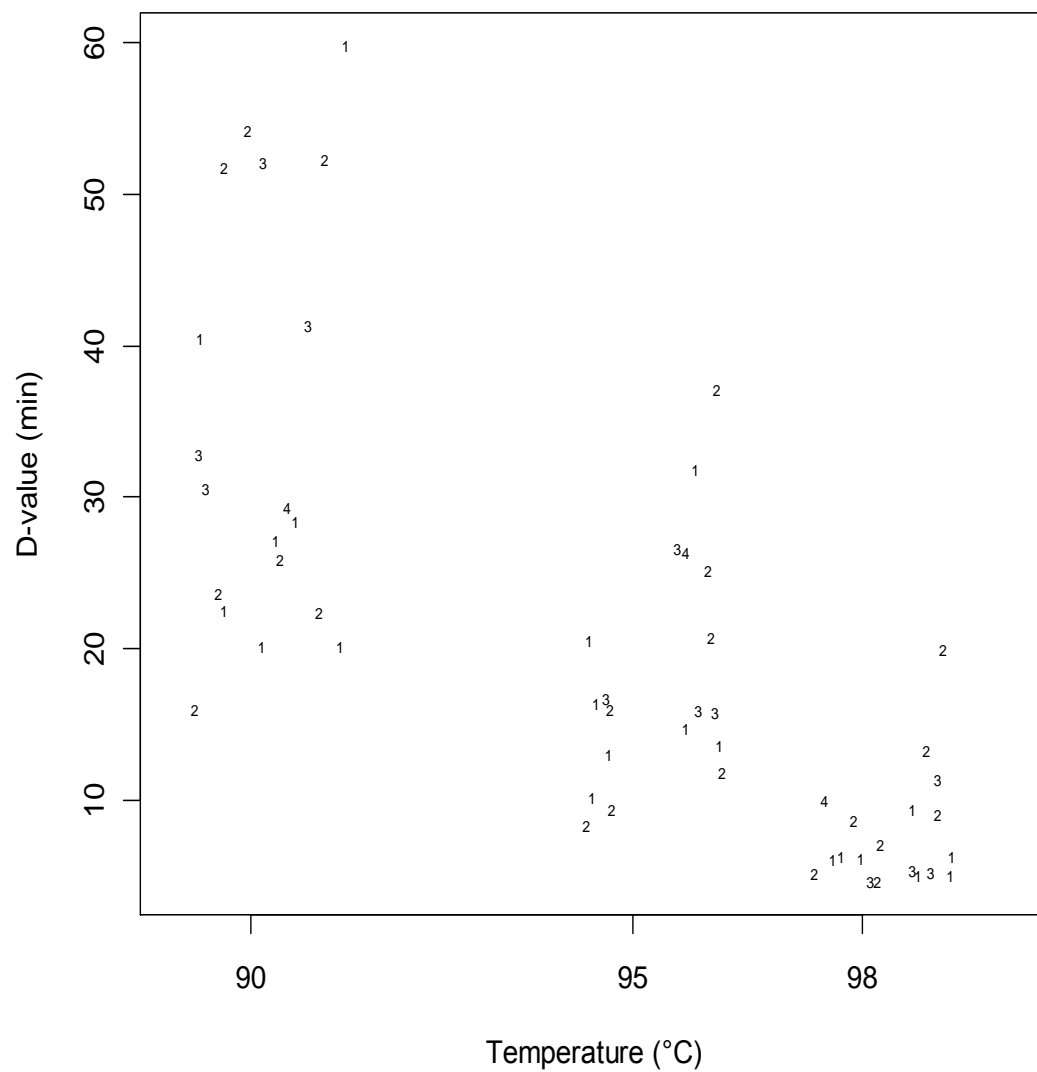


Figure 6.9. Scatter plot showing groupings of *dnaK* ATs and heat resistance (D_{90} , D_{95} , and D_{98}) of *A. acidoterrestris* and *A. acidocaldarius* strains

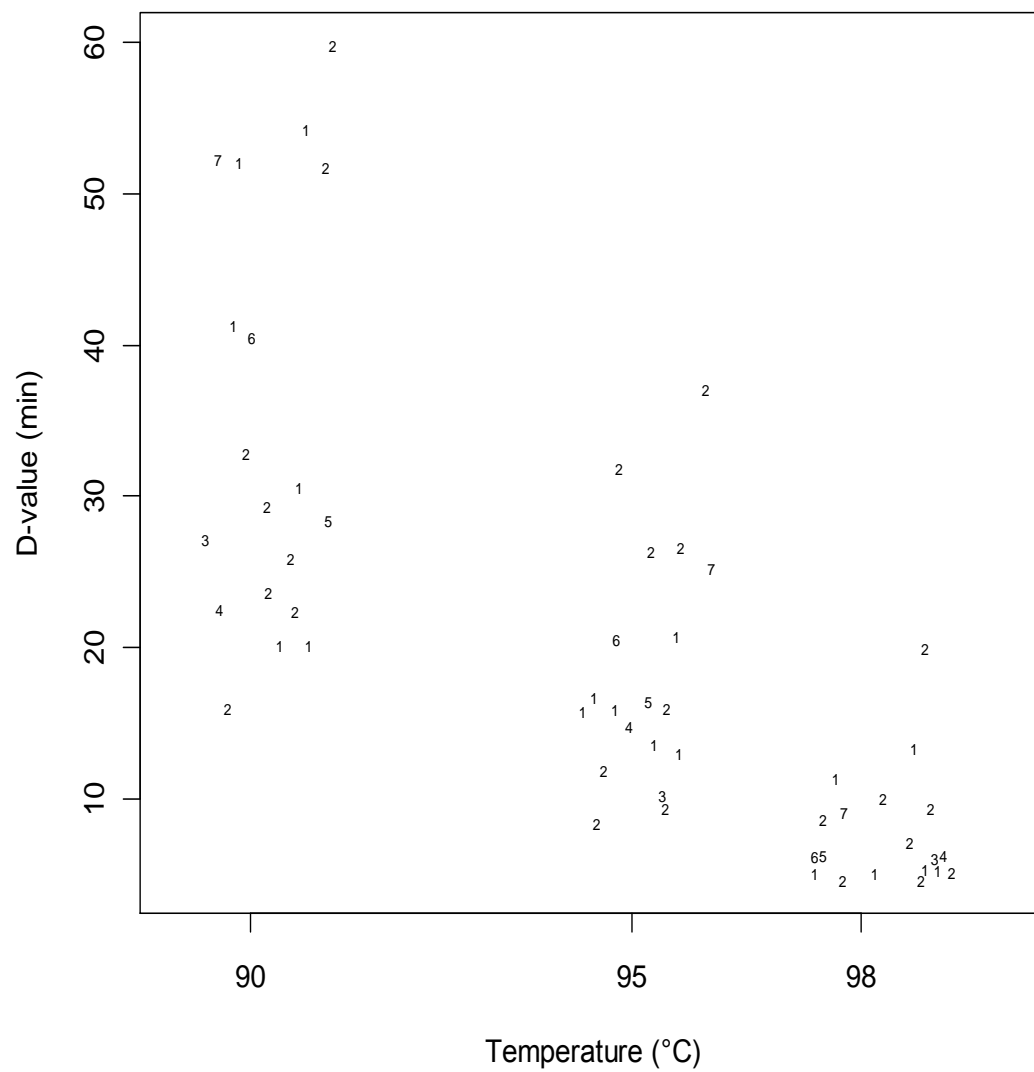


Figure 6.10. Scatter plot shows groupings of *rpoB* ATs and heat resistance (D_{90} , D_{95} , and D_{98}) of *A. acidoterrestris* and *A. acidocaldarius* strains

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CHAPTER 7

CONCLUSION AND DISCUSSIONS

The most important intervention method for the elimination or significant reduction of foodborne pathogens and/or spoilage microorganisms on fruits, vegetables, fruit and vegetable juices is strict adherence to Good Agricultural Practices (GAPs), Good Manufacturing Practices (GMPs), Hazard Analysis Critical Control Points (HACCP), and other relevant commodity-specific food safety and quality guidelines and recommendations. These recommendations cover all aspects of growing, harvesting, packing, transportation, processing, distribution, and preparation of raw fruits and vegetables to ensure safe, high-quality, products. However, even when recommendations are followed, it is almost impossible to completely prevent the introduction of foodborne pathogens and spoilage microorganisms to fruits and vegetables throughout entire production and processing cycles. Therefore, it is important to understand interactions between pathogens and produce and to develop and integrate successful intervention methods to reduce the risk of contamination of fruits, vegetables and juice beverages. Results of the current study demonstrated that optimized treatment with a combination of ultra-violet (UV) light (125 mJ/cm² or 500 mJ/cm²), acidified sodium chlorite (ASC; 200 ppm), and mild heat, 50°C) reduced *E. coli* O157:H7 populations as high as 6 log CFU/spot and 2.8 log CFU/spot on spot-inoculated green onions and baby spinach, respectively. Using the same optimized combination of UV, ASC, and mild heat, *E. coli* O157:H7 populations were reduced by 2.4 log CFU/g and 2.6 log CFU/g on dip-inoculated green onions and baby spinach, respectively. While optimized combined treatments of UV, ASC, and mild heat were found to be highly effective on surface contamination of green onions, they

were not as effective as on spot/surface decontamination baby spinach which indicates the necessity of development produce specific decontamination methods and parameters. On the other hand, these results indicated that this approach was of limited efficacy against pathogens on both green onions and spinach which were surface attached, protected, or infiltrated.

The use of gaseous ethyl pyruvate (EP) as a decontamination agent was also investigated in the current research. Treatment of dip-inoculated green onions with 400 $\mu\text{L/L}$ EP reduced *E. coli* O157:H7 populations by 5.2 log CFU/g after 7 days of storage at 4°C and by 5.2 log CFU/g after 5 days of storage at 10°C. Treatment of dip-inoculated baby spinach with 400 $\mu\text{L/L}$ EP reduced *E. coli* O157:H7 populations by 4.3 log CFU/g after 7 days of storage at 4°C and by 7.0 log CFU/g after 5 days of storage at 10°C. Taken together, these results indicate that gaseous EP has the potential to enhance the safety of fresh produce. Further research is needed to understand the mechanism(s) of *E. coli* inhibition by EP. The current study is the first time ever report on the use of EP as an antimicrobial agent on any fruits and vegetables.

Alicyclobacillus spp., a soil-borne, spore-forming bacterium, is one of the main spoilage organisms associated with off-odor development in fruit juices and beverages (1-3). In the current research, 141 *Alicyclobacillus* isolates collected from juices, beverages, ingredients, as environmental samples from were analyzed using 16s RNA gene sequencing. The most frequently isolated *Alicyclobacillus* spp. was *A. acidoterrestris* (45% of isolates). *A. acidocaldarius* subsp. *acidocaldarius* comprised 30% of isolates and *A. acidocaldarius* comprised 11% of isolates. Because of the high prevalence of *A. acidoterrestris* found in the in apple juice and concentrates sampled,

18 of the *A. acidoterrestris* isolates were further characterized with respect to heat resistance, guaiacol production, and *rpoB*, and *dnaK* gene analysis. Of the 18 isolates, 5 strains (CU83, CU87, CU88, VF, SAC) were highly heat resistant ($D_{90^{\circ}\text{C}} = 59.8, 51.8, 54.3, 52.1, 52.3$ min; $z\text{-values} = 10.4, 20.1, 13.0, 8.1, 10.8^{\circ}\text{C}$). All of the 18 *A. acidoterrestris* strains analyzed produced high amounts of guaiacol after 48 h incubation at 50°C , although strains varied in their rate of guaiacol production during the first 10 h of incubation at 50°C . Gene-based allelic type characterizations (16s rRNA, *dnaK*, and *rpoB*) indicated there was no correlation between genetic properties, guaiacol production rates, and thermal resistance. The analysis of more strains and/or improved molecular subtyping tools may be needed in order to better characterize the genetics and phenotypic properties *A. acidoterrestris*. However, the characterization of *A. acidoterrestris* isolated from apple juice and concentrates of the current study will help apple juice and beverage processors develop thermal processing regimes for apple juice and concentrates that provide protection against *Alicyclobacillus*-associated spoilage while maintaining product quality.

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